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PB-248 632

Toxicology of Anticholinesterase Pesticides

Yugoslav Academy of Sciences and Arts, Zagreb

Prepared for

Health Effects Research Lab, Research Triangle Park, N C

Jun 77

EPA-600/1-77-031

June 1977

PD 268 652

Environmental Health Effects Research Series



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TECHNICAL REPORT DATA

(Please read instructions on the reverse before completing)

1. REPORT NO. EPA-600/1-77-031	4. RECIPIENT'S AGENCY NO.
2. TITLE AND SUBTITLE Toxicology of Anticholinesterase Pesticides	5. REPORT DATE June 1977
3. AUTHOR(s) Elsa Reiner	6. PERFORMING ORGANIZATION CODE
7. PERFORMING ORGANIZATION NAME AND ADDRESS Institute for Medical Research and Occupational Health Zagreb Yugoslavia	8. PERFORMING ORGANIZATION REPORT NO.
9. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711	10. PROGRAM ELEMENT NO. 11A615
	11. CONTRACT/GRANT NO. SFCP-PR-2-616-2
	12. TYPE OF REPORT AND PERIOD COVERED
	13. SPONSORING AGENCY CODE EPA 600/11

14. SUPPLEMENTARY NOTES

15. ABSTRACT

The effect of pesticides was studied in humans and animal models, and on enzymes in order to develop a better understanding of their mechanism of action, and thus provide guidance for their safe use. It was shown that reduction of human blood cholinesterase activity is a good indicator of exposure to anticholinesterase pesticides. Regular measurements of blood cholinesterase activity and removal of individuals from suspected occupational environments prevented hazardous absorption of pesticides in occupationally exposed people. Ophthalmological studies indicated some functional eye impairment in people exposed to pesticides for many years.

The effect of one organophosphate ester used as a pesticide (trichlorfon; Metrifonate[®]) was studied in detail in humans, animals (rats) and enzymes (cholinesterases). It was shown that trichlorfon does not inhibit mammalian cholinesterases; inhibition of the enzyme is caused by dichlorvos formed from trichlorfon. The mechanism of reaction of acetylcholinesterase and cholinesterase with other inhibitors and with substrates was also studied in order to evaluate to what extent sites other than the catalytic sites are involved in the binding of ligands. Gas chromatographic methods for measuring pesticide residues were used to determine the concentrations of chlorinated hydrocarbon pesticides in the blood of the general population in Yugoslavia and for studies of organophosphate residues in urine.

16. KEY WORDS AND DOCUMENT ANALYSIS

17. DESCRIPTORS	18. IDENTIFIERS/OPEN ENDED TERMS	19. CIGATI Field/Group
toxicity cholinesterase inhibitors pesticides	trichlorfon Metrifonate	06 F

20. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	21. SECURITY CLASS (THIS REPORT) UNCLASSIFIED 22. SECURITY CLASS (THIS PAGE) UNCLASSIFIED	23. NO. OF PAGES 178 24. PRICE PCAO7 / MF / A01
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EPA-600/1-77-031
June 1977

TOXICOLOGY OF ANTICHOLINESTERASE PESTICIDES

by

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Special Foreign Currency Program
PR-2-515-2

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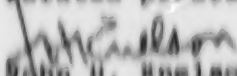
This report has been reviewed by the Health Effects Research Laboratory, RTP, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The use of pesticides to protect man and his environment has increased substantially over the past few decades. Considerable efforts have been made to assure the safe use of these toxicants both to the occupationally exposed and to those who are inadvertently exposed either directly or as a result of pesticide residues in food or the environment. Studies are reported on the health aspects of pesticides with the aid of volunteers from occupational exposure situations. Reports on adverse effects on ophthalmological parameters of certain organophosphate esters used as pesticides was evaluated using people occupationally exposed. The significance of these effects were evaluated and the continued surveillance of the population under substantial risk continues with emphasis on other health related problems.


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Director
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ABSTRACT

The effect of pesticides was studied in humans and animal models, and on enzymes in order to develop a better understanding of their mechanism of action, and thus provide guidance for their safe use.

It was shown that reduction of human blood cholinesterase activity is a good indicator of exposure to anticholinesterase pesticides. Regular measurements of blood cholinesterase activity and removal of individuals from suspected occupational environments prevented hazardous absorption of pesticides in occupationally exposed people. Ophthalmological studies indicated some functional eye impairment in people exposed to pesticides for many years.

The effect of one organophosphate ester used as a pesticide (trichlorfon; Metrifonate®) was studied in detail in humans, animals (rats) and enzymes (cholinesterases). It was shown that trichlorfon does not inhibit mammalian cholinesterases; inhibition of the enzyme is caused by dichlorvos formed from trichlorfon. The mechanism of reaction of acetylcholinesterase and cholinesterase with other inhibitors and with substrates was also studied in order to evaluate to what extent sites other than the catalytic sites are involved in the binding of ligands.

Gas chromatographic methods for measuring pesticide residues were used to determine concentrations of chlorinated hydrocarbon pesticides in the blood of the general population in Yugoslavia and for initial studies of organophosphate pesticide residues in the urine of occupationally exposed workers. Thin layer chromatography (combined with densitometric measurements of the chromatograms) served to determine organophosphates in experimental animals.

This report was submitted in fulfillment of a Special Foreign Currency Program (No. PR 2-515-2) by the Institute for Medical Research and Occupational Health (Zagreb, Yugoslavia) under the sponsorship of the U. S. Environmental Protection Agency. The report covers a period from June 1, 1972, to May 31, 1976, and work was completed on May 31, 1976.

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ACKNOWLEDGMENTS

The work presented in this report was performed by the following staff of the Institute for Medical Research and Occupational Health (Zagreb):

Senior scientists: R. Pleština (M.D., Ph. D.), V. Simeon (Ph. D.), M. Skrinjaric-Spoljar (Ph. D.), S. Stefanac (Ph. D.) and R. Wilhelm (Ph. D.)

Junior scientists: V. Bradamante-Simid (M.D., M.S.), R. Fink (B. S.), D. Kobrehel (M.S.), B. Krauthacker (M.S.), D. Mrvca (B.S.), B. Radić (M.S.), M. Stipcević (B.S.), S. Vasilid (B.S.) and S. Sime (M.S.)

Technicians: A. Buntić, A. Černik, T. Fajdetić, J. Kapetan, M. Kralj, M. Matasin, B. Thalcević and B. Volarić

Consultants from two other institutions also participated in the work: V. Turko (M.D.) from the Chemical works "Chromos" (Zagreb), and S. Horvat (M.D.), I. Ledić (M.D., Ph. D.), M. Pejić (M.D.) and M. Plukević-Pleština (M.D.) from the Department of Ophthalmology, University Hospital (Zagreb).

SECTION I

INTRODUCTION

Anticholinesterase agents are being extensively used as pesticides in agriculture, industry and for human health on a worldwide basis. The results presented in this report have been obtained over a period of four years by the team of workers listed in Acknowledgments. The report is divided into sections, each of which is written as a self-contained entity except for the references which follow the last section.

Sections 2 and 3 contain the main conclusions and recommendations for further studies.

Sections 4 through 7 report results of research on the effect of pesticides on humans. Section 8 describes in vivo studies on animal models while Sections 9 and 10 summarize the in vitro studies on enzyme mechanisms. Sections 11 and 12 deal with analytical chemistry and biochemistry methods for determining the effect of pesticides. Appendices A and B reflect two subjects not included in the EPA program, but which are related to its subject. An international meeting on cholinesterases and cholinergic receptors, organized in 1975, is discussed in Appendix A. Appendix B presents summaries of eight D.S. and M.S. theses related to the present report which were supervised by the senior members of our group; this activity arises from the fact that the Institute for Medical Research and Occupational Health is also a teaching institution of the University of Zagreb. Finally, the Reference Section gives a list of publications pertinent to the subject of this report including our own which have been published since 1972. The list includes all of our publications up to June 1976, but does not include manuscripts submitted for publication.

Several studies described in this report are a continuation of work started before 1972. Those studies described in Sections 5, 6, and 7 were initiated in cooperation with EPA and are now included in the Institute's long term research program. These studies also reflect the interest of the World Health Organization, of which organization this Institute is a Collaborating Center for Pesticide Toxicology.

SECTION 2

CONCLUSIONS

Human blood cholinesterase activity is a good indicator of the degree of absorption of anticholinesterase compounds, provided that preexposure or baseline enzyme activities were determined in the same subject before as well as after exposure. This was demonstrated in 485 individuals occupationally exposed to pesticides. The mean preexposure activities of a given population could not be taken as control values for an individual or a particular subject because of individual variations in cholinesterase activities. Signs and/or symptoms of poisoning by organophosphate anticholinesterase agents were always accompanied by inhibition of blood cholinesterase activity; the reverse was not the case.

The presence of metabolites of organophosphate esters in urine of occupationally exposed individuals is an adequate index of exposure. In order to obtain direct evidence of pesticide absorption, gas chromatographic methods were introduced for determining pesticide residues in humans. From initial studies of organophosphate residue analysis measurable amounts of a phosalone metabolite were present in the urine of 12 out of 13 workers occupationally exposed to phosalone. Blood samples from 147 individuals not exposed to pesticides all contained p,p' DDE; five other chlorinated hydrocarbons were also found in some of the blood samples.

Excessive exposure to pesticides over a period of several years seems to cause some functional eye impairment; narrowing of the visual field was more frequently observed in a group of 43 exposed workers than in a group of 20 nonexposed subjects.

The organophosphorus compound trichlorfon, which is a pesticide and also an antiparasitic drug (Metrifonate®), causes inhibition of cholinesterases when administered to humans or animals. It was shown by *in vitro* and *in vivo* studies that inhibition of the enzyme is not caused by trichlorfon itself, but that it is due to dichlorvos formed from trichlorfon by a nonenzymic rearrangement reaction.

SECTION 3

RECOMMENDATIONS

In subjects occupationally exposed to anticholinesterase pesticides, blood cholinesterase activities should be measured at regular time intervals in order to establish whether or not hazardous absorption had occurred and to monitor exposure. The colorimetric method developed by Ellman et al. (1961) is recommended as the most suitable for measuring cholinesterase activities; the method should be further developed and made applicable for field uses. In order to obtain direct evidence of pesticide exposure, residue analysis should be carried out; the available analytical methods for determining residues of cholinesterase inhibitors in humans need to be improved.

There is a need for a better understanding of the mechanism of action of chlorinated hydrocarbons. So far, direct measurement of residues in humans is the only means by which absorption is determined. Studies on the body burden of chlorinated hydrocarbons, and its relation to possible lesions, should be continued.

In individuals chronically exposed to pesticides, ophthalmological studies are required to establish whether or not the observed functional eye impairment is due to pesticides. Measurements of blood vitamin A levels should also be carried out.

Neurophysiological and behavioral studies should be initiated in people occupationally exposed to pesticides in order to establish whether or not pesticides diminish human functional capabilities.

Studies of the mechanism of action of pesticides on a molecular level should continue in order to gain a better understanding of their effects on humans.

SECTION 4

BLOOD CHOLINESTERASE ACTIVITIES IN THE GENERAL POPULATION AND IN WORKERS EXPOSED TO PESTICIDES

The need for effective pest control is well recognized. Pesticides are therefore necessary, and their economic value and efficacy in agriculture, industry, and disease prevention are unquestioned.

The acute toxicity of most organophosphorus esters used as insecticides is mainly, if not entirely, due to their inhibition of cholinesterases. As a result of cholinesterase inhibition in the nervous system, acetylcholine molecules accumulate at the synapse, initially causing excessive excitation and later leading to the blockage of synaptic transmission (Holmstedt 1959). At present, the only practical way to evaluate the hazards of over-exposure to anticholinesterase pesticides is to measure the blood cholinesterase activities in exposed people. In order to determine the condition which precedes clinical poisoning, an assessment was made of cholinesterase activities in the blood of workers employed in the production and application of pesticides. An attempt was made to correlate the activity of this enzyme with exposure and with the cholinergic signs and symptoms of poisoning.

The knowledge of the normal baseline blood cholinesterase activity in the human, and its variation is a necessary qualification for an interpretation of the results. Many investigations have been undertaken to survey blood cholinesterase activities of normal populations, that is, of groups of individuals that have not been exposed to cholinesterase inhibitors. Data in the literature were comprehensively reviewed by Calley et al. (1951), Kane (1958), Augustinsson (1955) and Gars (1967). Individual results were published by Humiston and Wright (1967) and Voss and Sachse (1970). The results can be compared only by calculation of the coefficient of variation, because the activities are expressed in different units according to the method of assay.

The assay of blood cholinesterase activity is fairly simple (Witter, 1963; Augustinsson 1971) and has been used for a number of years to assess the extent of human exposure (Hayes 1971; Sandifer et al. 1972; Warnick and Carter 1972). Such measurements provide a valuable confirmation of the diagnosis of

organophosphorus poisoning, since the early signs of intoxication with these substances are rather nonspecific. These measurements also permit the process of recovery to be followed, and support the decision of whether or not the patient is fit to return to work. Also, regular determination of blood cholinesterase may act as an early warning system for the recognition of excessive exposure.

Material and methods

Occupationally exposed workers were almost simultaneously exposed to various organophosphorus or carbamate esters as active substances. The list of compounds is given in Table 1.

The measurements of blood cholinesterase activity were performed in the laboratory, while the sampling was done either in the laboratory or in the field in which case the samples were kept cool and transferred to the laboratory. Cholinesterase activity was measured by the spectrophotometric method of Ellman et al. (1961) as modified for blood and plasma (Wilhelm 1968).

The reaction mixture contained 3.0 ml of phosphate buffer (0.1 M, pH 7.4), 100 μ l of the thiol reagent (0.01 M 5,5-dithiobis-3-nitrobenzoic acid) and enzyme preparation (20 μ l of plasma or 5 μ l of whole blood). When the substrate was added (50 μ l acetylthiocholine, final concentration 1.0 mM), the absorbance was read in 1.0 cm cuvettes at 412 m μ in the first and third minute after the addition of substrate, against the blank, which contained no substrate.

Cholinesterase activity was expressed in μ moles of thiocholine per min per ml, or in arbitrary units as Δ absorbance per min per ml of whole blood or plasma.

Under the conditions described, the activity of whole blood cholinesterase measured by this method is primarily due to the activity of erythrocyte cholinesterase. If it is assumed that the hematocrit is 50%, erythrocyte cholinesterase contributes 92% and plasma cholinesterase only 8% to the total whole blood activity in uninhibited samples (Wilhelm et al. 1973).

Whenever possible measurements of enzyme activity were performed before exposure and these values were taken as 100% in relation to activity determined after exposure. If that was not possible, 100% cholinesterase activity was determined much later, i.e. 2-3 months after exposure to any anticholinesterase compound. The value so obtained was then considered as the control value.

Blood samples were taken by finger-prick. Special care was taken to remove the insecticide from the skin by washing the hands with soap and water and rubbing the finger tip thoroughly with cotton-wool soaked in ethanol.

For determining plasma cholinesterase activity samples were collected in dry, heparinized, glass capillaries as described by Stubbs and Fales (1960). Plasma was separated by centrifuging the capillaries for 10 min. When necessary, the nondiluted samples were transferred to glass containers and kept in a vacuum flask with ice.

For determining whole blood cholinesterase activity 20 μ l of blood was collected in the laboratory or in the field, in Sahli pipettes which had been heparinized by rinsing with a 2% heparin solution immediately before the collection of samples. If necessary, the blood samples were then transferred, without dilution, into a glass container which was kept in a vacuum flask with ice. The same was done with the capillaries containing plasma.

The blood samples were collected from industrial workers at intervals which depended on the rate of production. When the production rate was very high, the sampling was done every second week.

In workers applying anticholinesterase insecticides in farms or orchards, blood cholinesterase activities were measured monthly; before, in the middle, and at the end of the working season.

Clinical Examinations, Blood Analyses

Safety-at-work regulations in Yugoslavia require that all workers employed in the production of pesticides submit to a continuous health control. This is accomplished in the factory health unit which is headed by a specialist in occupational medicine. Health control includes physical examinations twice a year. From the available medical records a picture of the general health condition of workers was formed.

The workers engaged in pesticide application are not medically surveyed continuously because of the seasonal character of their work. The general condition of their health was estimated from anamnestic data recorded at the beginning of a working season.

RESULTS AND DISCUSSION

Variation in Enzyme Activity within a Population

For the study of the distribution of normal cholinesterase activities, blood samples were taken from 400 healthy individuals with no previous exposure to anticholinesterase compounds. Many of them were later employed in the production and/or application of anticholinesterase insecticides so that their preexposure values served as control. The testing was done over a period of two years. In this way the distribution included possible external factors which might have influenced normal values.

The distribution of plasma and whole blood cholinesterase activity is shown in Figure 1. Enzyme activity is expressed in μ moles of thiocholine per min per ml, and ranged from 0.27 to 2.6 for plasma, and from 2.7 to 7.7 for whole blood cholinesterase. The estimated means were 1.3 and 5.1 respectively.

There is a slight overlap between the highest values of cholinesterase activities in the plasma and the lowest values in whole blood. Whole blood cholinesterase activities are distributed over a much wider range than those in the plasma.

In order to compare our results with those of other investigators who used different techniques, we calculated the results with respect to the coefficients of variation ($S.D. \times 100/\text{Mean}$). These were 18% for erythrocyte and 23% for plasma cholinesterase. This is in accordance with the results of other authors, who report coefficients of 10-15% for erythrocyte and 15-25% for plasma cholinesterase (Augustinsson 1955; Gage 1967; Voss and Sachse 1970).

Figure 2 shows that the distribution of values of plasma and whole blood cholinesterase activity for males differs from that for females. The difference between the means of the male and female groups is statistically significant for $t = 4.62$. The difference in the activity of the whole blood cholinesterase is probably caused by the difference in the hematocrit values which is due to sex (Augustinsson 1955).

To find out whether the activities of the two enzymes in man are mutually dependent we statistically analysed the values of the normal distribution.

The whole blood cholinesterase activity (y) was found to correlate well with the serum cholinesterase activity (x). The relationship between the two activities can be described by the empirical equation $y = (13.0 \pm 0.7) + (1.5 \pm 0.1)x$. This correlation was found valid for 400 samples.

The precision of the method was tested on a set of samples by two subsequent measurements of the activity of individual samples. The correlation between two pairs of measurements was $r = 0.99$ for plasma cholinesterase and $r = 0.91$ for the whole blood cholinesterase activities; such a high correlation permits a precise determination of sample activity by only one measurement.

From the results of other authors as well as from those reported it is evident that the measurements of preexposure enzyme values are indispensable before an individual is allowed to come into contact with anticholinesterase compounds. Because of a very wide range of normal activities great errors are possible in evaluating to what extent, if any, the examined person was exposed to an anticholinesterase compound. A subject who normally has a high average cholinesterase value might suffer a reduction which is due to his occupation, and yet this might not be recognized if the measurement referred to the population distribution, as it might well fall within the normal range of values. Conversely, the measurements made on an individual not occupationally exposed but with a genetically controlled atypical low plasma cholinesterase activity, Goedde et al. (1967) might give rise to unnecessary concern.

Variation in Enzyme Activity within an Individual

The degree of individual variation was studied in 34 subjects by simultaneous measurements of cholinesterase activities in plasma and whole blood. The successive measurements took place over a period of 15 months. They were performed in healthy adults selected among factory, office and research workers with no previous exposure to anticholinesterase compounds.

The measurements were carried out under conditions as identical as possible. All testing (sampling and measuring) was done by the same person using the same equipment. The factors of variation were 5.95 for the activity of whole blood cholinesterase and 8.77 for the activity of plasma cholinesterase.

Cholinesterase activity measurements were carried out over the period of 15 months. The temperature of the buffer ranged from 18 to 25°C. For the temperature correction factors the data published by Reiner et al. (1974) were used. Coefficients of variation agree with the data of other authors; these were 7.2 for whole blood cholinesterase and 11.9 for plasma cholinesterase activities.

The number of individuals in each of series of measurements varied between 28-34 except in the last measurement when only five subjects were tested.

Some of the individual curves show a slow progressive decline of activity towards the summer months, but in most of them this is interrupted by more abrupt variations. The data were analyzed to ascertain whether the differences between the results in successive measurements were lesser than predicted from the overall intra-individual standard deviation; a slight reduction was found in the order of ten percent. The differences between successive determinations in some cases exceeded 20 percent. The same differences were observed by other authors (Fryer et al. 1955; Gage 1955). All subjects were in good health and it was unlikely that they were suffering from unsuspected disease and disorders which might have affected blood cholinesterase activity.

Enzyme Activity in Workers from Pesticide Manufacture

Two groups of exposed workers were used in the study. The first group were workers from a manufacturer of anticholinesterase pesticides, and the second, workers from a pesticide application service.

A total of 356 industrial workers who worked in three different production lines in one plant were followed: 157 were engaged in formulating insecticidal dust or wettable powder, 136 formulated emulsions and 41 persons worked in the production of household sprays. In addition, 22 persons working in the laboratory were also studied.

The workers were aged 21-60 years. Only 85 were continuously employed in the production of pesticides for a number of years (2-10). The others were seasonal workers employed only during the periods of intense production.

The normal workday was eight hours, but periodically, because of intense production, overtime, up to 12 hours, was worked. The process of production in the three lines differs, so that the type of exposure was also different.

The production of emulsions involves a complex technological process, and there is a constant danger of workers coming in contact with active substances or final products, particularly because the air is contaminated with solvent vapors and active insecticidal substances. Workers have no fixed place, but move about as they work. In this study, the workers were most frequently exposed to phosalone, dimethoate, and dichlorvos.

The formulation of insecticidal dust is automated but in spite of that workers are exposed to pesticides. Active ingredients as a dust can settle onto their hands, clothes, and hair causing increased exposure. The compounds formulated were mainly monomethyl carbamates; diazocarb and carbaryl and only occasionally, and then for very short time intervals, the organophosphorus compound, phosalone.

In the manufacture of household sprays, dichlorvos and bromophos were produced. The workers handled very low concentrations of active substances, and exposure was relatively low.

In another plant, 83 workers were engaged in the production of pesticides containing thioneton or dichlorvos as the active material. Fifty-eight workers were employed continuously for five or more years while the remaining 25 seasonal workers worked only for about a three month period during peak production. In this plant, the same workers were involved in both the production of emulsions and the manufacture of household sprays. Work conditions did not differ from those above.

The demands for insecticides imposed by the market, especially in spring and summer months, have resulted in an increase in production. Therefore, in the first six months of the year workers are more heavily exposed to organophosphorus and carbamate insecticides.

Tables 2, 3, and 4 show the average number of days of exposure and a list of active insecticidal substances to which a particular worker was exposed in the period from 1971 until 1975. Days of exposure mostly relate to the first half of the year.

Table 5 shows the average number of overtime work hours for each worker in the production of emulsions in 1971 and 1972.

The results of measurements of cholinesterase activity in the blood in exposed workers from all the three production lines are given in Table 6. The symptoms of poisoning are recorded with relative enzyme activities.

In order to establish a relationship between exposure and enzyme activity, the data of the group of workers involved in emulsion production were analysed over several years. Figure 1 shows the mean values of cholinesterase activity in the blood and plasma for the whole group ($N = 11$), and also the duration of exposure between measurements for each worker.

The production of organophosphorus insecticides increased between 1971 and 1975 as shown in Tables 2, 3, and 4. We expected that with the increase in exposure, the number of blood samples with enzyme activities less than 75% would also increase. However, Table 6 shows that the greatest number of blood samples with reduced cholinesterase activity was recorded in 1971. The accompanying symptoms or signs of poisoning were also most numerous in the same year.

In 1971 the workers were exposed to and handled mainly dimethoate and chlorfenvinphos, both very dangerous insecticides which are extremely toxic to mammals. Certain technological procedures and working conditions provided inadequate protection, and in addition there was a great deal of overtime work during the peak of the season (Table 5). Twenty-four workers had some complaints, and after the cases of poisoning were analysed working conditions were considerably improved before the 1972 season. Therefore, although the extent of work remained the same (Tables 2, 3, 4, and Figure 1) there were no cases of occupational poisoning in 1972, except for one accidental intoxication at work.

In 1973 the situation, as noted in 1972, did not change. Only two exposed workers had blood cholinesterase activities below 50% which is below the limit for occupational exposure. They were removed from further exposure. Four workers with moderate complaints underwent a systematic medical examination. Their blood cholinesterase activity was above 80% of the normal value and no signs of poisoning were found.

In the past two years (1974-1975) there has been an increase in the production of insecticides. The number of seasonal workers with no previous experience with insecticides also increased. From the results presented in Table 6, the number of reduced enzyme activities again seems to have become higher. If, however, the accompanying symptoms observed in some workers are expressed in percent, they are 29% in 1971, 0.95% in 1972, 9% in 1973, 17% in 1974, and 12% in 1975.

Table 7 related to the poisoning of a few workers during the period of observation. The poisonings were recorded mostly in 1971 (N = 7). Only those workers are listed for whom it was possible to ascertain that the accompanying symptoms were due to excessive exposure to anticholinesterase compounds. After a two week removal from the place of exposure cholinesterase activity increased in all these workers. The workers were exposed to various organophosphorus or even carbamate insecticides so that the spontaneous reactivation of the inhibited enzyme was not identical in all workers.

It is seen in Table 1 that the workers were exposed to organophosphorus compounds containing dimethoxy or a diethoxy radicals. The acetylcholinesterase of human erythrocytes inhibited by a dimethoxy organophosphorus compound is known to reactivate quickly ($t_{1/2}$ = 51 min) while for the plasma cholinesterase inhibited by the same group of organophosphorus compounds the half time reactivation is $\approx$ 83 hours (Skrinjaric et al. 1973); erythrocyte acetylcholinesterase inhibited by diethyl organophosphorus compounds also reactivates faster ($t_{1/2}$ = 2.4 days) than plasma cholinesterase ($t_{1/2}$ = 30 days) (Reiner 1971). Further, most organophosphorus insecticides inhibit, to a greater extent, plasma cholinesterase (Simson and Reiner 1973). The emulsion-production workers had a lowered plasma cholinesterase activity in most measurements which is in accordance with the literature data about the slow spontaneous reactivation and higher inhibition of serum cholinesterase. Still, we did not consider this as too important, for plasma cholinesterase can be highly inhibited without any consequence to health. In workers exposed to dimethoxy organophosphorus compounds such as dichlorvos, dimethoate and others, the phenomenon of aging of the inhibited human erythrocyte cholinesterase, should be borne in mind since it occurs relatively fast (half-time of aging about four hours) in contrast to diethyl organophosphorus compounds with a half-time of aging of about 40 hours (Aldridge and Reiner 1972). The aged cholinesterase is irreversibly inhibited and cannot be reactivated even by oximes.

In workers exposed to carbamate insecticides, it was difficult to assess the actual degree of exposure because of the already well known property of carbamylated cholinesterase to reactivate spontaneously in a very short time (Wilson et al. 1960; Reiner 1971). Reiner and Aldridge (1967) showed that the half-time of reactivation for monomethyl carbamates is 30 minutes, for dimethyl carbamates only 76 minutes if the cholinesterase of purified bovine erythrocytes was carbamylated. Some of the workers from the production of insecticidal powder had blood cholinesterase activities less than 60% at the time of sampling, but a 24-hour interruption of exposure was sufficient for cholinesterase activity to return to normal. Since the duration of exposure had no lasting effect on cholinesterase activity and on workers' health, the cholinesterase activities in these workers were monitored only 2-3 times a year, during the peak of production.

In order to determine the degree of exposure in the course of one working day, we measured blood and plasma cholinesterase activities in workers before and after the work shift. The results are presented in Table 2. In ten workers from the group engaged in the formulation and packing of phosalone, cholinesterase activity was measured at 8 a.m. and at 1 p.m. After

cessation of exposure in five workers the measured blood cholinesterase activity was the same, while in the other five it was reduced by 6-10%. Plasma cholinesterase activity in these workers was reduced by 10-15%.

In another pesticide formulating plant, workers were engaged in the production of pesticides containing thiometon or dichlorvos. Since for most of the workers no preexposure values were recorded, it was not possible to calculate the actual degree of cholinesterase inhibition. However, in comparison with the normal values determined by the same method in nonexposed workers enzyme activities were sometimes low. Only the tendency of a further recovery or diminution of enzyme activity could be registered.

The results of measurements of enzymes activities in the workers whose preexposure values were taken are presented in Table 9. The mean activities of a group of workers engaged in the formulation of thiometon or dichlorvos are given for a period of six years. Very often enzyme activities were low. Almost always these low values were accompanied by symptoms of poisoning such as headache, fatigue, giddiness and sweating.

Workers Employed in the Application of Insecticides in Agriculture

To assess the extent of exposure in workers who apply anticholinesterase insecticides, we chose 129 agricultural workers engaged in orcharding (41) or farming (88). Their jobs are seasonal and last usually about two months. The working conditions were different from those of industrial workers described earlier. These individuals work in the open where the possibility of contamination is smaller. Also, they come into contact with formulated or finished products and not with the active pesticide ingredient. However, the exposure can increase due to windy weather or high temperature.

The workers who sprayed farms were exposed mainly to 5% phorate, and to a lesser extent to diazinon, methidathion, fenitrothion, and parathion. The exposure in 1973 was about 10 working days over a period of two months. In 1974 and 1975 the average exposure was 16 days.

After recording preexposure values for each work year in the course of the working season, in 1973, cholinesterase activity was measured twice, while in the following years enzyme activity was determined only once during the exposure period. The results of measurements are given in Table 10. In the first year of testing, the group as a whole did not show very reduced

mean values of cholinesterase activity. The lowest blood cholinesterase activity was 84% of the preexposure value.

In the second year, of 44 tested workers, six had blood cholinesterase activity between 60% and 70%, six between 70% and 80% and the rest over 80%. This decrease indicated that the workers absorbed a certain amount of organophosphorus insecticides in the course of work.

The group of orchard spraymen was exposed to a greater variety of compounds. In one farm in 1973 phorate (5%), parathion (46.5%), diazinon (20%) and fenitrothion (10%) were applied. The results are given in Table 11. Work conditions were poor - the work was not mechanized and protective devices were used infrequently. The exposure to anticholinesterase compounds was about 60 days over four months. The measurements were done after approximately 20 days of exposure. Cholinesterase activities showed no significant trend to decrease, although the lowest activity was obtained in the third measurement (Mean = 86% for blood cholinesterase and 75% for plasma cholinesterase). In 1975, the exposure of people employed in another orchard which used Azinphos-methyl (25%), phosalone (30%) and carbaryl (50%) as insecticides was followed. The agricultural work was completely mechanized except in places where the mixing and diluting of the compounds took place. The workers were exposed to anticholinesterase compounds continuously for only two to three days with fortnight breaks. Table 11 shows that their cholinesterase was practically undiminished, particularly when data are compared with the former group.

The exposure of workers employed in the agricultural application of insecticides is essentially different from that of industrial workers. Work is done in the open with interruptions, and finished products are used.

Four of eight examined workers employed in the orchard spraying (1971) showed and reported some signs and symptoms of poisoning, such as sweating, headache, and nausea. The same symptoms were observed in farm spraymen whose cholinesterase activity was below 70%. Although the symptoms were not typical cholinergic symptoms, it was concluded they were due to the absorption of a certain amount of anticholinesterase compound. The workers were in good health and the symptoms cannot be ascribed to any other cause.

Protection at Work

As protective clothing, industrial workers wore only canvas clothes, rubber gloves and rubber shoes. For face and eye protection they occasionally used face shields and goggles, but no respirators or vapor filters.

Agricultural workers wore only protective clothing, and periodically gloves and respirators. In some orchards the spraying operation was highly mechanized. However, neither the drivers nor the mixers observed protective measures such as wearing gloves or a respirator. The mixing of concentrate was done in a most inappropriate way.

Blood Picture and Exposure to Organophosphorus Compounds

According to literature data (Davignon et al., 1965; Paccagnella et al., 1971) it was expected that increased exposure of workers employed in the production of organophosphorus insecticides would reduce the total number of leukocytes, and increase the number of lymphocytes in response to a possible allergic reaction of the worker to the insecticide in question.

In nine workers who worked in a pesticide industry for a number of years a differential blood analysis was made. In addition, the total number of leukocytes and the number of lymphocytes per 100 leukocytes was measured in the course of their work with anticholinesterase insecticides. During intense exposure to these compounds (1970-1974) 51 analyses were carried out. In the same workers, 50 further analyses were done between the years 1966 and 1969 when they were not in contact with anticholinesterases. Ninety-seven differential blood analyses were done in 48 transportation workers who never had been in contact with pesticides.

The results are shown in Table 12. No significant differences in the same workers were noted in the period before and after exposure to organophosphorus compounds. Also, no differences were observed between the exposed group and the control group. The results agree with those of Warnick and Carter (1972), and Ensborg et al. (1974), but do not agree with those of Davignon et al. (1965) and Paccagnella et al. (1971).

SUMMARY

Occupationally-exposed individuals who work in the formulation of anticholinesterase insecticides are exposed to high concentrations of active substances at their place of work. Working in a confined space, periodically intense production, and difficult working conditions all contribute to excessive absorption of these compounds. By the use of prescribed protective devices at work, and observing the hygienic and sanitary regulations, workers can achieve a satisfactory degree of protection and thus reduce the possibility of contact with anticholinesterase insecticides.

Regular measurement of cholinesterase activity in these workers in the course of the work season, i.e., once or twice in a fortnight during the period of intense production, is presently the only suitable method for the determination of the degree of absorption of an anticholinesterase insecticide at work. With this method one can learn in time whether there are workers at risk whose enzyme activity is lower than their pre-exposure value, and thus prevent the danger of further absorption. In all workers under study a significant drop in blood cholinesterase activities could always be associated with excessive exposure due to overtime work and/or lack of protective measures. However, this does not apply vice versa, i.e., excessive contacts with anticholinesterases did not always lead to a significant drop in blood cholinesterase activities. Without exception, workers showing cholinergic symptoms had their blood cholinesterase inhibited equal to or greater than 50%. However, complaints from workers were not always related to cholinesterase inhibition.

Working conditions of those who apply insecticides in agriculture are different from the conditions in insecticide production. Working in the open is an important factor which helped to reduce the absorption of anticholinesterase insecticides, and only bad atmospheric conditions, uncontrolled handling of these final products, and poor protection at work could result in excessive absorption. Treating the workers as a group, we did not find more than about 10% cholinesterase inhibition, although a few individuals showed inhibition up to 40% and these also had subjective complaints. In workers exposed to the production of organophosphorus insecticides, the analysis of differential blood pictures revealed no change in the leukocyte count nor in the number of lymphocytes. We believe, therefore, that these compounds have no toxic effect on the blood of exposed workers.

Since enzyme activities vary within a population as well as within an individual, it is important to establish normal values for a given population. Plasma and whole blood cholinesterase activity was determined by a spectrophotometric method in a group of 400 healthy human subjects (257 males and 151 females) with no previous exposure to anticholinesterase compounds. The results of enzyme activities (expressed in μ moles of thiocholine/min/ml) were distributed over a comparatively large range: 0.35 - 2.45 for plasma, and 3.09 - 8.05 for blood cholinesterase, with a mean of 1.34 and 5.17 respectively.

Variations within an individual were assessed in 34 subjects (14 males and 20 females) by measuring cholinesterase activity in plasma and whole blood every 4-5 weeks over a period of 15 months. The cholinesterase activity fluctuated during that period with the mean coefficient of variation of 11.9 for plasma and 7.2 for whole blood. In the summer months, the cholinesterase activities showed a slight tendency to decrease.

SECTION 5

EFFECT OF PESTICIDES ON EYE AND SIGHT

This section contains two parts: a review of the literature and the results of current investigations.

The literature review, while not a critical review, was intended to collect all available information pertinent to the topic. Conclusions were taken from authors' summaries or abstracts as published largely in tertiary publications. The source of information is always quoted as the primary reference with a notation of secondary or tertiary sources as needed. In order to make the review as comprehensive as possible, some references are inserted which are not discussed. Those which had a very general abstract are marked with an asterisk. When the same results were published in more than one paper, only one paper was reviewed; references to the other papers are marked with an asterisk. Papers which were not accessible, even as a summary or abstract, are marked with two asterisks.

REVIEW OF THE LITERATURE

For the most part, knowledge of the biological effects of acute exposure to pesticides has been well documented. The same cannot be said for the effects of chronic exposure. At one period it was assumed that the only threat from pesticides was through by their acute toxic effects. Recently, however, a number of authors suggest different nonspecific effects of various pesticides due to chronic exposure including anticholinesterases.

There is a vast quantity of literature on the various effects of pesticides in experimental animals, many of which have not been confirmed in humans. For this reason, increased emphasis on research using human subjects has been recommended by many researchers, organizations, and government agencies (U.S. Department of Health, Education and Welfare 1969).

Among many untoward effects of pesticides used in public health programs and in agriculture, visual impairment has very recently begun to receive more attention. Since the beginning of this decade a number of reports have appeared primarily in the Japanese literature describing a visual impairment in

people, including occupationally exposed, the general population, and children in particular.

Records indicate that Japan is one of the most polluted countries in the world (Ui, 1972) with one of the world's highest utilization rates of organophosphorus pesticides per unit area (Kogure and Imai, 1974) and a corresponding large number of yearly poisonings. A decade ago Dr. Ken Ohto noticed an unusual eye disease in children and adults in the Saku locale, Nagano Prefecture, in Central Japan. The disease became known as the Saku disease. The incidence of the disease increased at a high rate in many other regions in Japan (Ishikawa and Ohto 1972). The same or a similar disease, however, as a special nosologic entity, has not been recognized in other parts of the world.

The first systemic investigations were started in 1969 by Ishikawa and his coworkers (Ishikawa et al. 1970; Ishikawa 1971a). From ocular and general signs of poisoning or exposure, Ishikawa concluded that the Saku disease was due to chronic exposure to organophosphorus pesticides (Ishikawa 1971a). The features of eye impairment, as "one of the most consistent symptoms of chronic poisoning in people living in agricultural areas," gradually became very striking (Ueno 1972; Kogure and Imai 1974). On the basis of an official report submitted to the Japanese Government, the use of parathion, EPN, TEPP and malathion was banned and their replacement by less dangerous pesticides among organophosphorus compounds was encouraged. Ishikawa (1974) extensively summarized the results of investigations in the Saku region, tabulating diagnostic criteria, ocular findings, blood cholinesterase activities and residues of organophosphorus compounds, both in exposed people and control subjects.

Standards for ocular anomalies attributed to organophosphorus pesticides (Kato 1972) set up by the Japanese Ministry of Public Welfare include: impaired vision (less than 1.0 after correction), visual field stenosis (less than 40 degrees nasal and less than 60 degrees temporal), progressive myopia, serious astigmatism (difference between horizontal and vertical radius larger than 2.0 D), impaired sense of balance (standing time on one leg less than five seconds), effective cholinesterase reactivator (2-PAM or Padrin) therapy, lowered cholinesterase activity, and edema and atrophy of the optic nerve (pale in color in the temporal side of papilla). If five out of the above eight conditions were positive the diagnosis of the eye damage by organophosphorus compounds was made (Ishikawa 1972a, 1972b).

The suggestion that the etiology of the disease was associated with exposure to organophosphorus pesticides was taken for granted. As such, some clinicians started to diagnose every obscure eye impairment in violated areas by measuring blood cholinesterase activity (Saito and Kuba 1972). Moreover, the therapy was established along these lines, and patients were

treated with oximes and atropine for many months (Ichii and Kimata 1973; Ohto 1974; Osawa et al. 1973). The improvement of the clinical picture following the oxime therapy is sometimes regarded as confirming a diagnosis of chronic poisoning with anticholinesterase (Ohto 1971). Oishi (1974), however, warns that unless the pollutant is definitely known, ophthalmologists should not place a diagnosis such as postbulbar neuritis or toxic amblyopia, after only a few examinations.

Some authors suggest that organophosphorus compounds might affect other sensory systems apart from vision. Osawa et al. (1975) showed that in patients with chronic organophosphorus intoxication, the sense of balance can also be disturbed. Besides eye abnormalities Kwalick (1971) found a significant difference in the number of individuals with hearing problems comparing pesticide-exposed subjects with controls. Fuji et al. (1975) reported on disturbed vestibular function in addition to other nervous symptoms and visual impairment in subjects who were in contact with pesticides.

Epidemiological Studies

Epidemiological studies were conducted on occupationally exposed workers and on the general population, mostly children, living in the areas where insecticides were used extensively. An extensive retrospective investigation, conducted in several areas in Japan, analyzed the relationship between the use of organophosphorus compounds and incidence of myopia in approximately 40,000 school children over a period of 16 years (Tamura and Aitsui, 1975). A positive correlation was found between the amount of organophosphorus pesticides used in a specific year and the incidence of myopia in the following year. A monitoring of the refractive condition of the eyes revealed that the subjects from the city of Saku showed a tendency to become myopic (Otsuka et al. 1972). The axial length of the eye of the Saku subjects was inclined to be shorter than that of the controls. It was found that the lower the serum cholinesterase activity, the greater the axial length. However, there was no significant correlation between the amount of organophosphorus residues in the sera and the axial length of the eye. Development of myopia caused by chronic organophosphate pesticide intoxication has been reviewed recently (Ishikawa 1976).

A large ophthalmological trial in which visual field screening included 11,151 school children living in the region where insecticides were extensively used revealed visual abnormalities in 32 children. In only two cases no explanation for the abnormality was found (Futemma 1973). According to the investigation of a large number of school boys, the relationship between the symptoms of ocular anomalies (set in diagnostic

standards for ocular anomalies attributable to organophosphorus pesticides) and the extent of exposure was so obscure to be almost irrelevant (Kato 1972). Also in a much larger study on 30,000 pupils, the results did not prove any statistically significant difference in ophthalmological findings between the rural and urban population (Kato 1973).

However, eye abnormalities in school children living in violated agricultural areas were observed by other authors. In an ophthalmological survey on 141 nursery school children living in an agricultural area where organophosphorus insecticides were repeatedly detected in appreciable amounts from house dust, Mitsui (1972) found abnormalities in eyesight. This included: refraction in 19, narrowing of the visual fields in 26 and abnormal eyeground in 8 subjects. Tsukahara (1972) examined 90 children, 10 of whom were chronically exposed to pesticides. In these children visual acuity could not be corrected with lenses, and in most of them, papillae were slightly congested or discolored. Electroretinography revealed no abnormalities. Among one hundred randomly selected school children between eight and nine years of age, five to seven percent was found to have ocular symptoms, either ocular neuritis, constricted visual fields or astigmatism (Ishikawa et al. 1971). In almost all of the twenty-two children (six to twelve years of age) with eye disorders attributed to the exposure to organophosphorus compounds, ophthalmological examination revealed remarkable constriction of visual fields (Takaku 1972).

In Japan and in other countries there were several retrospective studies in agricultural workers handling pesticides for a various lengths of time. From ophthalmological examination of 1,995 agricultural workers and 2,372 students of agricultural technology, Bugnini (1971) concluded that organophosphorus insecticides, to which the workers were mostly exposed, produced an alteration in the intracocular pressure and in some other parameters. He pointed out several contraindications concerning the eye and vision for the subjects who intended to work in different agricultural activities including the handling of agricultural chemicals.

Ophthalmological examinations of 164 Japanese agricultural workers, handling pesticides for over 30 years, revealed a considerable number of functional eye impairments (Imaiume et al. 1971). A similar examination was performed earlier in the Soviet Union (Medvedovskaya 1967) in a total of 776 agricultural workers divided into six groups according to the type of work. The incidence of eye impairment was highest in group of 60 workers whose only job was to handle pesticides (44.8% versus 25.1 to 41.1% in other groups). In another study conducted by the same author (Medvedovskaya and Golenka 1971) in 33 female workers highly exposed to pesticides, eye disease was found in

36% mainly located in the anterior portion of the eye. Refraction anomalies and degenerative alterations of the conjunctiva (pinguecula and pterygium) were the most frequent findings. In 412 farmers handling chemical poisons, a high incidence of corneal abnormalities and glaucoma was found (Barya 1973). Nuritdinova (1973) also described an extremely high incidence of the pathological eye findings among 240 occupationally exposed workers. In another clinical trial on 693 occupationally exposed subjects, eye abnormalities were found in 90% (Nuritdinova 1975). The most frequent findings were dystrophic changes of the iris and narrowing of the visual fields. A variety of systemic, neurological and ocular symptoms were reported for 51 adult patients who complained of blurred vision (Ohto 1971). Ocular symptoms included swollen disks, spasms of the ciliary muscle, cataract and disturbance of the smooth pursuit motion of the eye. In the 60 heavily exposed pesticide workers, no biochemical abnormalities were noted (Pietsch et al. 1972). Ophthalmological examinations of these workers revealed a few cataracts and a high incidence of cortical wedging, as compared with unexposed subjects. Agricultural workers handling chloroethene complained of epiphora and photophobia and had very pronounced conjunctivitis (Sivitskaya and Alekseevich 1973). On detailed examination, constricted visual fields and decreased eye pressure were observed. The workers handling thiram (tetramethylthiuram disulfide, TMTD) also had, conjunctivitis, as well as slightly decreased eye pressure and decreased corneal sensitivity (Sivitskaya 1974).

However the results were not positive in all epidemiological investigations. A health survey of twelve male professional pesticide applicators who worked with fenitrothion and dichlorvos for two to nine years revealed no ophthalmological abnormalities (Mizumi et al. 1975). In a retrospective study on 114 persons who were obviously poisoned by organophosphorus insecticides eight complained of sight impairment, six of them blaming insecticides as the cause of their troubles (Tabershaw and Cooper 1960). However, the physician in charge thought it was of a different etiology than organophosphate pesticide poisoning or exposure.

Complaints received from workers exposed to pesticides were predominantly those of visual acuity disturbance, photophobia and epiphora (Medvedevskaya and Golenko 1971; Imazumi et al. 1971; Hanyu and Tsui 1974; Ito 1973). Ophthalmological examination of the frontal segment frequently revealed conjunctival hyperemia (Kalič-Filipović et al. 1973; Sivitskaya 1974; Sivitskaya and Alekseevich 1973; Hanyu and Tsui 1974; Glaske 1970; Medvedevskaya and Golenko 1971). Corneal ulcerations were also mentioned (Imazumi and Atsumi 1971). In 21% of 200 pesticide applicators a new formation of corneal vessels was found (Ito 1973). In these patients serum vitamin B₂ was significantly lower.

The occurrence of glaucoma was frequent among occupationally exposed workers (Medvedovskaya 1967; Nuriddinova 1973; Sarya 1973; Sivitskaya and Alekseevuk 1973). The narrowing of visual fields the most frequently report abnormality, was first described by Ishikawa (1971a) who found a concentric narrowing in all examined Saku children. Increased incidence of visual field anomalies was reported as in occupationally exposed adults both in Japan as well as in occupationally exposed adults both in Japan and in some other countries (Ishikawa et al. 1970, 1971; Imaizumi et al. 1971; Nekamura et al. 1971; Futenma 1973; Mitsui 1972; Takaku 1972; Gaisko 1970; Nuriddinova 1973, 1975; Sivitskaya and Alekseevuk 1973). Visual field examinations were performed on 19 children presumed to be victims of organophosphorus poisoning, on ten normal adults, and on five healthy children (Ohba et al. 1972). In spite of obvious differences in findings the authors insist on setting up the standards for visual field parameters in normal children before making a meaningful comparison. A study of the visual field of 15 pilots and two other persons taken before, during and after occupational exposure to TEPP did not reveal any meaningful change (Upholt et al. 1956). Other functional eye tests were only seldom applied. Abnormal electroretinographic (ERG) tracings were found in known cases of intoxication by organophosphorus insecticides (Imai et al. 1971a). Disturbances of dark adaptation time was mentioned by several authors (Nuriddinova 1973; Kalid-Filipović et al. 1973; Gaisko 1970). Experimental studies of this problem were reported by Rubin and Goldberg (1957, 1958). They found that sarin, an anticholinesterase compound, when inhaled by volunteers significantly elevated the absolute visual threshold for up to 24 hours after exposure. When sarin was applied topically to the conjunctival sac of the eye, a similar effect was not seen (Rubin et al. 1957). It was therefore concluded that sarin brought about visual threshold through an extracocular action, probably in the central nervous system.

Disturbed smooth pursuit motion of the eyeballs in more than half of the examined subjects was reported by Ishikawa (1971a). Similar findings were also reported by other authors (Fuji et al. 1975).

Several authors noticed abnormal pupillary reaction, but only Ishikawa (1971a) used videopupillographic technique in order to quantify the observed abnormalities. He found that exposed subjects and controls significantly differ in the rate of reaction of light stimuli.

Some epidemiological data discussed above are summarized in Table 13. In some reports, however, chronic exposure is confused with chronic poisoning. The ophthalmological and biochemical methods used in the reports are only seldom described in sufficient details to reproduce the results. The criteria for the diagnosis of eye impairment are not always comparable. The

interpretation of the results described in the Table 13 should therefore be made very carefully. However, it should be emphasized that the incidence of different eye damage in examined subjects varies according to these reports between zero and a hundred percent.

Case Reports

A 67 year old patient who sprayed malathion and fenitrothion for many years complained of gradual deterioration of vision (Ishikawa 1972a). Ophthalmological examination revealed a loss and narrowing of the vision, anomalous eye movement and irregular size of the pupil. These changes disappeared after the treatment with PAM.

In two adult patients with chronic organophosphorus poisoning (Gono et al. 1973) corneal astigmatism, myopia and constriction of visual fields were found. The symptoms gradually returned to normal after five months of therapy with PAM. Similarly the main symptoms in three children and one adult (disturbance of vision, peripheral structure of visual field, abnormal refraction, disturbance of eyeball motion) were attributed by the author to the exposure to anticholinesterase insecticides and were treated as such (Ohto 1972).

Ophthalmological examination of twelve children with chronic organophosphorus intoxication (Ohto 1974) revealed apart from abnormalities of the papilla (congestion and paleness) a constriction of the visual fields. Electroretinographic examination revealed low amplitudes of α - and β -waves.

Campbell (1952) described several cases of polyneuritis in people who had been using insecticides. In three out of seven cases retrobulbar neuritis was also present. However the author was reluctant to claim that the findings were associated only with the proven exposure to DDT. Besides myopathy, which developed in two agricultural workers exposed to organophosphorus compounds, a 47 year old mother and 22 year old son, annular abnormalities were present around macula lutea with pigment deposits (Kamoto et al. 1973). Bilateral optic neuritis was diagnosed in a 47 year old subject (Uchida 1974). He had retinal choroidal degeneration, and also suffered from neurosyphilis and diabetes. However, he frequently used malathion and since visual disturbances improved after a therapy with PAM, the case was considered to be a poisoning with organophosphates.

A rare transient bilateral internal ophthalmoplegia was observed in an adult with a history of exposure to a commercial insecticide containing pyrethrins which suggested a possibility of a toxic etiology of the disease (Weintraub, 1971). Twelve hospitalized patients treated for eye impairment claimed by the

author (Ohta 1974) to have been caused by pesticides were found to suffer from progressive pigmentary degeneration without any family connection. Five cases of upward gaze paresis or paralysis combined with optico-autonomic neuropathy have been described in pesticide applicators who had handled different organophosphorus pesticides for at least five years (Ishikawa et al. 1976).

Untoward Effects of Anticholinesterases Used as Drugs in Ophthalmological Treatment

Exactly one century has elapsed since physostigmine was introduced as the first anticholinesterase drug for the medical treatment of glaucoma. A variety of different acetylcholinesterase inhibitors have been used since for the treatment of this disease. With the use of prescribed concentrations, applied locally to the eye, adequate control of glaucoma can be maintained generally without the occurrence of side effects. If these occur, however, they are obviously of systemic cholinergic stimulation through cholinesterase inhibition or are most likely associated with this mechanism (Ellis 1966).

However, local application of anticholinesterase miotics in glaucoma treatment caused lenticular changes in a number of patients (Axelsson and Holmberg 1966; DeRoeth 1966; Axelsson 1968b; Abraham and Teller 1969) but not in guinea pigs (Axelsson 1971). In human subjects occupationally exposed to anticholinesterase agent, lenticular changes have been described only seldom (Pietsch et al. 1972; Komoto et al. 1973). The application of miotics which are of carbamic acid origin, although anticholinesterases, have never caused lenticular opacities (Axelsson 1968a).

The mechanism by which these agents produce cataracts is still unclear. The presence of lenticular cholinesterase as well as its inhibition by anticholinesterase miotics were clearly demonstrated (DeRoeth 1966; Laties 1969). However, it seems that there is no direct relationship between inhibition of cholinesterases and obstruction of lens clarity (Michen and Kinoshita 1968). The cataracts initiated by anticholinesterase agents applied topically are specific in type (Shaffer and Hetherington 1966) if compared with common cataracts seen in elderly people. The cataractogenic activity of different chemical agents including anticholinesterase drugs has been adequately reviewed (Paterson 1971; Gehring 1971).

A case report (Hornblase et al. 1974) deals with topical application of thiopropazone which caused a delayed side effect - periorbital skin depigmentation. The mechanism of depigmentation is completely unclear.

Studies on Experimental Animals

Different abnormalities in chronic organophosphorus intoxication of experimental animals observed by a number of authors point out the predominance of sensory neuropathy as compared with affection of the motor nerve system. Other authors however found no eye and sight abnormalities in animals treated with certain anticholinesterases which are frequently blamed for causing eye damage in man. Thus Nishimura and coworkers treated beagles with fenitrothion 20 mg/kg/day for 10 months and on ophthalmological examinations found no difference from control animals in many of the parameters measured (Nishimura et al., 1974). Neither did Ogata (1972a) find any meaningful clinical or pathophysiological anomaly in dogs treated with the same or similar compound. Blindness in cattle in some areas in Japan was formerly attributed to a direct effect of anticholinesterases. However, Ohishi (1972) regards it to be connected with vitamin A deficiency, since serum and liver carotene was found to be extremely low as was serum and liver vitamin A.

Morphological and Biochemical Changes

Severe ocular abnormalities, such as turbidity, protuberance and projection of cornea were found in rats fed on a diet containing up to 600 ppm of fenitrothion (Ooka et al. 1973). However, although marked inflammatory changes were seen in all corneal layers by light and electron microscopy, no significant changes were observed in the retina or in the optic nerve. The incidence of corneal opacities produced by this compound or similar compounds, could be appreciably reduced by subcutaneous application of glutathione (Hikita and Ishikawa 1972). In another experiment, besides atropine and PAM, Naite et al (1973) tested glutathione in the therapy of corneal abnormalities in rats given organophosphorus compounds and found that glutathione was not therapeutic. In rabbits sprayed with a high concentration of fenitrothion a decrease in the radius of corneal curvature was observed (Kawai and Naite 1974). The same was noticed in beagles treated with a dose of 5 mg/kg/day fenitrothion during 10 months (Kawamura et al. 1974). The corneal curvature was considerably reduced until growth stopped. Even smaller doses produced similar effects (Ueda et al. 1972).

Myopia appeared in beagles only four months after beginning the application of ethylthiometon at a dose of 5 to 15 mg/day/dog (Tokoro 1973). Astigmatism was also present and the findings resembled the clinical picture reported in children from the Naku District. In the experiment carried out by Suzuki and Ishikawa (1974) all dogs treated with the same compound showed myopia after 12 months of administration. Myopia progressed until cessation of administration. Thiometon given to dogs at

a dose of 0.8 - 1.0 mg/day over a period of 100 days caused myopia in three out of five animals (Ishikawa 1972d). Other corneal abnormalities in experimental animals treated with different anticholinesterases, were described (Machashi et al, 1972; Naito et al, 1973a, 1973b).

The mechanism of formation of described corneal abnormalities is unknown. Corneal epithelium contains acetylcholine and the enzymes acetylcholinesterase and cholinacetyltransferase are very active (Howard and Wilson 1972) but whether these facts are in any way connected with the abnormalities reported in experimental animals and humans remains to be explained.

Myopia frequently observed in children and in experimental animals was correlated with the exposure to organophosphorus compounds. In thiometon treated beagles a significant inverse correlation between blood cholinesterase activity and the length of the optic axis was found (Otsuka 1971).

After a repeated administration of ethylthiometon to a beagle over a period of two years the basement membrane between ciliary muscle cells was extremely thickened as revealed by electron microscopy (Araki et al 1973). In an identical experiment the fine structure of the ciliary muscle of beagle dogs treated with ethylthiometon revealed unique membranous structures occupying the cytoplasm of one third of the muscle fibres (Suzuki and Ishikawa 1974). Structural changes of the ciliary muscle were also found in rabbits and monkeys treated with dichlorvos (Katsushima et al, 1973). Similarly, morphological changes in the extracocular muscles of beagles treated with ethylthiometon were described by Mukuno and Imai (1973). In monkeys and rabbits treated repeatedly with fenitrothion, motor and plates changed conspicuously especially in the outer ocular muscle (Matsushima et al, 1972). In the same animal species treated with dichlorvos or fenitrothion Yamasaki and coworkers reported alteration of the junctional fold and synaptic vesicle, a swelling and a decrease in the number of mitochondria in the motor and plates of the external eye muscle (Yamasaki et al, 1975). These results suggest a possibility of the chronic progressive paralysis of extracocular muscles in man.

Microscopical changes in the optic nerve fibers were found in beagles treated with thiometon for two years (Kano et al, 1975). A dose response relationship was observed with respect to the ratio of myelinated nerve fibres. A similar compound produced axonal denaturation of the optic nerve in the beagle, so that the mechanism of action of organophosphorus compounds on eye might be connected with the inhibition of protein synthesis and repression of axoplasmic flow (Mukuno et al, 1973; Mukuno and Ishikawa 1975). A histopathological study of the canine optic nerve and retina was undertaken to investigate morphologically and quantitatively the structural changes caused by repeated

administration of ethyithioneton (Uga et al, 1976). The optic nerve was found to be invariably affected and the number of myelinated axons was markedly reduced. Changes in the retina were mainly seen in the pigment epithelium.

In rabbits fed on a diet containing fenitrothion significant changes in the composition of fatty acids in the optic nerve were found (Imaizumi et al, 1973). The same compound produced retinal changes in puppies (Homma 1973). At the beginning of administration, the reddening of the papilla and retina and a dilatation of the central vein of the retina were found. Discoloration of the papilla and retina was seen after a long time.

Functional Changes.

Retinal function has been extensively studied but little is known about how it is impaired by chemical agents, pesticides in particular.

To explain some functional anomalies observed in experimental animals Miyata and coworkers studied localization of cholinesterases in rat's eye (Miyata et al, 1974). Acetylcholinesterase was found to be present in the retina and cholinesterase in the pigmentary epithelial layer. By histochemical techniques, cholinesterase was found to be only slightly inhibited after the administration of 5 mg/kg of fenthion and completely inhibited after 100 mg/kg.

Electroretinographic studies carried out in mice treated with parathion showed that the c-wave disappears quickly and that the latency time of the b-wave increases and its altitude decreases (Carricaburu and Laurein 1973). The electroretinographic measurements were also carried out in flies poisoned by parathion (Carricaburu, 1971). Retinal disturbances in rats given fenthion were recorded by electroretinography by many authors (Miyata et al, 1973; Imai 1974a, 1974b, 1975; Imai et al, 1973b). The maximum noneffect level was found to be very low, 5 µg/kg, i.e. 1/60,000 of the LD₅₀ dose of the compound (Imai 1974a). According to the same group of authors, the maximum noneffect of fenthion for the retina was estimated to correspond to 25 mg of the compound for a human subject (Imai et al, 1973b). The authors therefore conclude that ERG examinations should be performed in suspected poisonings by organophosphorus compounds.

There are very few reports which describe other functional tests in experimental animals. In an interesting experiment Lehner (1971) found no measurable effect on color vision in dieldrin treated ducks. Rats fed on a diet containing 50 ppm of disulphoton over a period of two months showed no performance deficit in tests in which depth perception and discrimination were required (Clark 1972).

Ophthalmological Examination of Workers Exposed to Pesticides

The purpose of our present work was to investigate the existence of morphological changes of the eye and functional eye impairment in workers exposed to pesticides, who had been under medical observation for many years and whose exposure was assessed in several ways. These were predominately the same workers who were taken into the study of the relationship of pesticide exposure and its effect on blood cholinesterases (Section 4). The ophthalmological studies were undertaken because there is no doubt that among many subjects (about 100,000) described in the literature at least some showed eye and visual impairment. The extent to which this is related to pesticides requires further elucidation.

Exposed Workers and Control Subjects

A total of 61 workers were taken in this study. They were divided into three groups according to the exposure to pesticides. Some data on the ophthalmologically examined workers are shown in Table 14.

Group A -- Consisting of 11 workers was selected according to the length of time each worker was engaged in pesticide production. Only those working longer than five years were taken in the study. All of them were well trained permanent staff, mostly from the production of pesticide emulsions.

Initially, the working conditions of these occupationally exposed to pesticides were rather poor, but they improved gradually. The active ingredients produced were predominantly, but not exclusively, anticholinesterase compounds, consisting mainly of different types of dimethoxy and diethoxy phosphates of thiophosphates and carbamates. A few compounds were chlorinated hydrocarbons or herbicides. Workers had no fixed working place and an irregular rotating system was introduced to equalize exposure. Usual working time was eight hours a day, five days a week for 9 - 10 months a year. However, overtime work was quite common, particularly when seasonal production had to be increased. A regular monitoring of cholinesterase activity showed an association between overtime work and decreased blood cholinesterase activity. The details concerning the extent of exposure, type of compounds and their effect on blood cholinesterases are described in Section 4.

Group B -- Among twelve workers, seven were engaged for more than five years in pesticide application, largely in public health programs, but also in disinfection of food stores. Two workers were pilots who applied pesticides in agriculture and

these remaining supervised pesticide production. A common factor for all the people in this group was irregular exposure, good protective measures and regime at work. All this made for a considerably milder exposure than for workers in group A. Again, the active materials were predominantly anticholinesterase compounds except for pilots who were equally exposed to different fertilizers and herbicides. Blood cholinesterase activity data, monitored over a long period of time, did not reveal any toxicologically meaningful pattern of enzyme inhibition among these workers. Similarly, other routine biochemical and hematological tests required by clinicians at their periodical medical checkup, were normal.

Group C -- This group of 20 subjects was formed to serve as control. The workers were recruited among those of similar socio-economic status as for the two previous groups. They were matched by age and habits, but had no greater contact with pesticides than the general population. They worked either in internal transport or in the storage of end products which were not insecticides. Thus except by sex, the three groups were matched as closely as possible.

Methods

The ophthalmological examinations took place at the Ophthalmology Department of the University Hospital, Zagreb Yugoslavia. Throughout the study, all examinations were performed by the same team of ophthalmologists. The examiners were not aware of workers' exposure. As a rule, in each subject all the examinations were completed within one or two consecutive days. However, whenever additional tests were needed, the workers were asked to come as many times as needed. The prerequisite condition was imposed that before any ophthalmological examination was made the worker must have felt healthy and be fit to work.

All the workers were questioned about previous eye or other relevant diseases, family history and present complaints concerning the eye and vision.

By external eye examination all changes on the eyelids, in the motility of eye-balls, bulbar and palpebral conjunctiva, cornea and lacrimal apparatus were recorded. Whenever necessary an examination by slit lamp microscopy (up to 64 x magnification) was performed.

The corneal curvature was determined with a keratometer which was either Javal or Bausch & Lomb. The results were calculated as differences in dioptries between the two meridians and as corneal curvature expressed in millimeter values.

Visual acuity was determined with the Snellen chart under standard illumination. The vision was expressed by a fraction in which the numerator denotes the distance at which the test was conducted (5 or 6 m) and the denominator the distance at which the smallest letters read subtend a visual angle of five minutes. Whenever possible, the refractive errors were corrected to as near normal sight as possible. In patients with discrepancy in the visual acuity and estimated refraction ability a scleroptic test was carried out after adequate cycloplegia. This was, however, always done after measuring of internal eye pressure but before perimetry. Testing of accommodation ability and focusing power was done with Jäger reading charts J₁ - J₄.

Measurement of the intraocular eye pressure was performed in all the workers with the same Schiøtz' tonometer. The eye pressure was expressed in millimeters of mercury after the results were calculated from the instrument's calibration graphs. In patients who showed even minimal abnormality in the pressure, the measurements were repeated several times. If again found abnormal, the patient was thoroughly investigated in order to exclude or confirm the diagnosis of glaucoma. Applanation tonometry and gonioscopy were standard tests carried out in these patients.

Ocular media and eyeground were examined after the pupil was widened with a short lasting mydriatic drug. Special attention was paid to estimate the transparency of ocular media.

Visual fields were examined in detail in all workers with a Goldmann perimeter 940 (Haag-Streit). This was done after visual acuity determination and if necessary after its appropriate correction by lenses. The basic illumination of the perimeter's calota was kept constant, 1,000 asb. The size of the target was also constant, being 0.35 mm², but its illumination varied. Relative intensity of the illuminated target was 1.00 (I₁), 0.315 (I₂), 0.10 (I₃) and 0.0315 (I₄) of the basic illumination. The examinations were carried out according to the principle "nonseen - seen" at 15° intervals. These values were later computed for each eye, each isopter and each of the three groups. The means were also calculated.

Dark adaptation was determined in most workers from the three groups on a Goldmann - Weekers adaptometer. The examined person was kept in absolute darkness for three minutes and then exposed to light of 2,000° asb. over five minutes. After that, the absolute threshold of excitation was determined at one and two minute intervals over the period of 35 minutes. The examined area was in the paracentral region 11° remote from the center of fixation. The target was a 100% contrast black-white striped plate with a 56 mm diameter. The method applied was again "nonseen - seen" i.e. starting with complete darkness, the illumination of the target was gradually increased until

the object was seen. The individual results obtained as adaptation curves were expressed in logarithmic values for each minute, and means calculated for each group.

Color vision was tested in two ways: with a dichromatic test according to several pseudoisochromatic tables (Ichikawa tables 1964; Velhagen, 23rd edition; Color Vision Test 1957) and with an anomaloscopic instrument according to Nagel, Type 1.

The test with pseudoisochromatic tables was carried out in daylight for each eye separately. The examined subject was not allowed to wear glasses while examined.

The examination with the Nagel, Type 1 anomaloscope was conducted by applying the Rayleigh equations. The anomalous quotient (AQ) was calculated from the results obtained.

Results and Discussion

The complaints received from workers exposed to pesticides (Groups A and B) were predominantly lacrimation, photophobia and a subjective feeling of burning and itching of the eyes. These complaints were not received from control subjects. The summary of complaints is shown in Table 15. Some complaints which were obviously not associated with exposure to pesticides, were disregarded.

The results of external eye examination are summarized in Table 16. By external eye examination conjunctival injection of different degrees was found in more than half of the examined workers from group A. This finding could explain frequent complaints received from exposed workers. However, in group B similar changes were not observed, while 8 out of 20 control subjects showed changes similar to those in group A. Dilated episcleral blood vessels were seen in many exposed subjects. Apart from the dilated episcleral blood vessels, which are not usually so frequent in the general population, the other findings were commonly recorded abnormalities, conjunctival infection being slightly more frequent in exposed workers.

In three patients from group A an irregular astigmatism was diagnosed. In one case the abnormality was in a very advanced stage. Otherwise, corneal curvature measurements revealed no striking differences in the groups tested. The difference between the two meridians (placed at 90°) was within the expected physiological difference (less or around 1 D). These values were not statistically different from control subjects. The results are shown in Table 17.

Visual acuity was determined in all workers and if found to be lower than 1.0 it was corrected by lenses to as near normal as possible. The results showing visual acuity of both eyes before and after correction with lenses are plotted in Table 18.

With very few exceptions the visual acuity which was lower than normal (1.0) could have been adequately corrected by lenses.

A rather rough method for testing accommodation ability and focusing power did not reveal any greater impairment.

The measurement of internal eye pressure by means of Schiotz' tonometer revealed values which are expected in this population. However, in workers exposed to pesticides the internal eye pressure was significantly lower ($P < 0.01$) if compared with control subjects. The results of these measurements are shown in Table 19. Although several workers had a considerably higher pressure possibly indicating glaucoma, applanation tonometry and gonioscopy confirmed this diagnosis in only one worker from Group A. This was an advanced stage of an open angle glaucoma which had not been recognized before. The results of this measurement were omitted from the table.

Ophthalmoscopic examination of the eyeground revealed only mild degenerative changes of the retina, mostly in elderly patients. In addition, in several patients retinal blood vessels were slightly altered, but the findings were not very striking. The findings are summarized in Table 20.

Opacities of optic media were found only in two workers from group A and in one from group B. In all others, and in group C, the optic media were absolutely clear.

The perimetric measurements revealed a significant narrowing of the visual field in the second and third isopter. The location of this defect is rather unusual and at present cannot be explained. The results expressed as mean values for each isopter and each group are shown separately for each eye in Tables 21 and 22. At several points the difference between the two isopters is statistically highly significant ($P < 0.001$). Since all the measurements were carried out on the same instrument, by the same person and under the same conditions there is little doubt that the differences were an artefact. However, it is obvious that the constriction of the visual field was very mild and therefore its pathophysiological meaning should be carefully considered.

Color vision testing showed no striking differences between the three groups tested. The anomaloscopic quotient (AQ) was found to be close to the normal physiological value in all workers.

Dark adaptation ability was slower in workers exposed to pesticides than in control subjects (Table 23). The difference became significant ($P < 0.05$) only at 15th minute, being considerably larger at 21st minute ($P < 0.001$).

Although some morphological changes and functional eye impairment were found more frequently in exposed workers than in control subjects, additional tests and a larger number of examined subjects are required before any firm conclusion on the effect of pesticides on eye and sight is made.

Summary

The effect of pesticides on eye and sight is comprehensively reviewed and 147 literature references are provided. Data were not reviewed in a critical way. The results of epidemiological studies on exposed humans conducted in several countries are tabulated (Table 13).

The results of our own investigations which were initiated recently are presented in ten tables. Ophthalmological examinations were carried out mainly in the workers described in Section 4. Sixty-three workers were examined of whom 20 served as controls.

The analysis of the results revealed the narrowing of the peripheral visual field (particularly in the second and third isopter of either eye) in exposed workers when compared with control subjects. The difference was significant ($P < 0.05 - 0.001$) in the lower temporal region. So far this finding has no explanation.

Internal eye pressure was found to be lower in exposed workers than in controls ($P < 0.01$).

The dark adaptation ability after 15 minutes was considerably slower than in controls ($P < 0.05 - 0.001$).

In other clinical parameters there were no great differences between exposed workers and control subjects.

SECTION 6

RESIDUES OF ORGANOPHOSPHORUS PESTICIDES IN HUMAN URINE

For guidelines to control occupational exposure to organophosphorus pesticides, the determination of organophosphorus residues has been suggested. For this reason, the method of Shafik et al. (1974) has been introduced. In this study, the determination in a group of 11 workers of one metabolite of phosalone is described.

Materials and Methods

Table 24 shows the names, abbreviations and formulas of compounds used in this work.

N-amyl-N'-nitro-N-nitrosoguanidine produced by Aldrich Chemical Co., Milwaukee, Wis. was obtained from the EPA Repository at Research Triangle Park, N. C., as were all standard substances and mixtures used. All other reagents were of the highest purity grade available, and the solvents were further purified.

The gas chromatograph, Varian Aerograph Model 2860, with Alkali Flame Ionization Detector (AFID) with Rb₂SO₄ salt tip was used. The gas chromatographic column used was: Boreasilicate glass, 6' x 1/4" o.d. packed with 4% SE-30 and 6% OV-210 Gas-Chrom Q 80/100 mesh.

The operating conditions for the gas chromatographic analysis, the sensitivity, and retention times are listed in Tables 25 and 26.

The following stages of the original procedure of Shafik et al. (1974) were tested with standard solutions:

Alkylation--Known amounts of dimethyl phosphoric acid were dissolved in hexane and the alkylation with diisopentane performed, omitting the step up by chromatography on silica gel columns.

The effects of various parameters were tested on the yield of the alkylation (Table 27):

- added amount of freshly prepared diasopentane;
- aging of diasopentane solutions (with 1 ml of diasopentane added);
- duration of the alkylation (with 1.5 ml of diasopentane added).

Alkylation and column chromatography--As already noted in the original procedure, the elution pattern in the chromatographic separation on silica gel columns may vary from one laboratory to another, indicating a need for establishing a standard elution pattern.

Using a 3 x 1 cm column of silica gel (2 g silica gel and 2 g Na_2SO_4) the impurities were eluted with 20 ml CH_2Cl_2 at the beginning of the separation. An additional volume of 20 ml CH_2Cl_2 with 3% of acetone contained the whole amount of DMAP and DEAP. This volume was evaporated to dryness and the residue dissolved in 5.0 ml of hexane for gas chromatographic analysis.

DEADTP was partially eluted with 3 ml of a solvent mixture CH_2Cl_2 + hexane (1 + 1), and an additional 3 ml contained the rest. Both fractions were united, evaporated to dryness and the residue dissolved in 2.0 ml of hexane for gas chromatographic analysis. Further fractions contained only the impurities. Mean values of the recovery in these stages are shown in Table 27.

Extraction, alkylation and column chromatography--The critical stages of the procedure - Extraction, alkylation and column chromatography - were tested by adding DMP, DEP and DEBTP to urine samples of unexposed subjects. The results are shown in Table 27. Recoveries calculated in Table 27 are based on the amount of standard added to the samples.

Results and Discussion

Urine (10 - 20 ml) was collected from workers industrially exposed in the formulation of phosalone. Samples were taken on a Tuesday at the end of the workday. From each sample, two aliquots of 2 ml each were analysed for DEBTP. To another two aliquots (2.0 ml each) DEBTP was added (900 ng per ml urine) in order to evaluate the recovery. The eluates from the silica gel columns were evaporated to dryness and dissolved in 2.0 ml hexane. For the gas chromatographic analysis 1.0 to 1.5 μl of the hexane solution were injected. The identification of the compound was based on the retention time as compared to known standards. The smallest amount detectable was 7 ng DEBTP per ml urine. The

obtained results are summarized in Tables 28 and 29; the tables also contain the results obtained on urine samples of 8 unexposed individuals.

In urine samples from five subjects not exposed to pesticides, the amount of DEADTP was < 7 ng/ml urine. The exception was subject V.B.S. who had < 7 ng/ml on one day, and 13 ng/ml several days later. In urine samples from workers formulating phosalone the amount of DEADTP was between 12 and 105 ng/ml urine. The exception was one worker whose urine contained < 7 ng/ml. Consequently, it seems that 12 out of 13 workers absorbed phosalone.

Blood cholinesterase activities were always measured in these workers at the beginning of the same workday as sample urine collection was made. All activities were within 85 to 100% of the preexposure value, except for subject I. S., whose whole blood cholinesterase activity was 73%, while the plasma activity was 100%.

The recovery of DEADTP added to urine samples varied from 12 to 32%, with a mean value of 21%. Such recoveries are low indicating that the method requires improvement. As some reagents used in the procedure are hazardous (nitrosequanidine and diisopentane) it would be advantageous to develop a more suitable method.

SUMMARY

The gas chromatographic method of Safik et al. was applied to measure one metabolite of phosalone in urine samples from 13 workers formulating phosalone. In 12 of the 13 workers measurable amounts of 0,0-diethyl phosphorodithioate (DEADTP) were found. The recovery of the procedure was tested by adding known amounts of DEADTP to urine, and it was shown that 21% of the added compound could be recovered.

SECTION 7

RESIDUES OF CHLORINATED HYDROCARBONS IN HUMAN BLOOD

There are no readily available data on the content of chlorinated hydrocarbon residues in blood of the population of Yugoslavia. The use of chlorinated hydrocarbons has recently been restricted, and it was of interest to initiate a survey which would establish the present levels and enable a later followup of the body burden of chlorinated hydrocarbon pesticides. A small number of individuals (147) in a continental town in Croatia was selected, and their plasma or serum were analysed for those pesticides which are still in use or were recently used.

Methods

The chlorinated hydrocarbons were determined by gas-liquid chromatography. The extraction of compounds was done within 24 hours after sampling, and the extraction procedure was according to Dale et al. (1970). From each individual two 1.0 ml samples (acidified with formic acid) were extracted with hexane (four 1 ml aliquots). The combined extracts were purified on a Florisil column (1 g Florisil + ca. 1 g anhydrous Na_2SO_4). The hexane was evaporated from the eluates, and the compounds were dissolved in 1.0 ml hexane. The concentrations of chlorinated hydrocarbons were determined on a Varian 2800 gas-liquid chromatograph with an H^3 - 80 electron capture detector. Three different column packings were used: Column A - 1.95% QF-1 and 1.5% OV-17 on Gas Chrom Q, 80-100 mesh; Column B - 4% SE-30 and 6% OV-210 on Gas Chrom Q, 80-100 mesh; Column C - 3% QF-1 and 6% DC-200 on Varaport 30. The best separation was obtained on Column B. On all columns, the compounds were identified by their retention times as compared with known standards. Solutions of standards in hexane were treated in the same way as the hexane extracts. The purification of extracts on Florisil column is not included into the procedure of Dale et al. (1970). The purification was necessary because fatty acids were found in the extract. It was shown that except for dieldrin, no loss of chlorinated hydrocarbons occurs by that procedure. The concentrations of dieldrin are therefore not quoted.

Results and Discussion

Samples of plasma (92) or serum (55) of 147 individuals were analysed for their content of chlorinated hydrocarbons. The individuals were 65 males and 82 females; their mean age was 42 years, ranging from 8 to 92 years. Blood samples were obtained from clinical laboratories during the year 1973. To our knowledge none of the individuals had a previous occupational or accidental exposure to chlorinated hydrocarbons or pesticides in general. The results are presented in Table 10. All samples contained p,p'-DDE, but p,p'-DDT was found in only 20 samples. In five (out of the 20 samples) the concentration of p,p'-DDT was higher than p,p'-DDE; in the other 15 samples the reverse was true. The mean ratio of p,p'-DDE/p,p'-DDT in these 20 samples was 2.3. Similar to p,p'-DDT, the other compounds were not found in all samples analysed. The concentration range for a given compound is large and the concentrations agree with published data for other populations (Davies 1972; Kolmodin-Hedman 1974).

Summary

Samples of plasma or serum of 147 individuals of a general population in a continental town in Croatia were analysed for chlorinated hydrocarbons. The compounds were determined by gas-liquid chromatography and identified by their retention times as compared with known standards. All samples contained p,p'-DDE (mean conc. 35.3 ppb), but p,p'-DDT was found in only 20 samples (mean conc. 22.7 ppb). The concentrations of the other compounds were 3.25, 4.09, 12.2 and 11.6 ppb for α -HCH, lindane, aldrin and p,p'-DDD respectively.

SECTION 8

TOXIC EFFECT OF METRIFONATE (TRICHLORFON) AND DDVP (DICHLORVOS) IN RATS

Metrifonate is the international nonproprietary name for the pharmaceutical substance 0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate. This compound was first prepared by Lorenz and coworkers (Lorenz et al. 1955) and was introduced by Bayer, West Germany in 1952. It has been commercially available since then. As a pesticide, with the common name trichlorfon, it has been recommended for use as follows: against flies and other household pests (WHO Expert Committee on Insecticides 1963), in agriculture (Gosldner 1960; Müller 1961), in veterinary practice as an antiparasitic drug in the treatment of various helminths, and for the control of ectoparasites (Boyd and Bullard 1960; Khan et al. 1972; Khan 1973). It is also known to be effective in the treatment of human *S. haematobium* infection (Davis and Bailey 1969; Flustina et al. 1973). Its toxicity to mammals is surprisingly low in comparison with other organophosphorus compounds. The rat oral LD₅₀ is approximately 600 mg/kg (DuBois and Cotter 1955; Gaines 1969). Insecticidal activity of trichlorfon is attributed to its conversion to dichlorvos (0,0-dimethyl 2,2-dichlorovinyl phosphate, DDVP) (Metcalf et al. 1959). However, DDVP has not been found in any appreciable amounts after the administration of trichlorfon to experimental animals (Robbins et al. 1956; Arthur and Casida 1957). According to some authors (Fukuto and Metcalf 1969), trichlorfon itself is devoid of anticholinesterase action but others (Arthur and Casida 1957; Hobbiger 1963; Hassan et al. 1965) regard it as a direct inhibitor of cholinesterase. The *in vitro* studies on that question are described in Section 9. DDVP is a well known and widely used insecticide, considerably more toxic for mammals than trichlorfon (Gaines 1969). It was also used as an antiparasitic drug (Chavarría et al. 1969; Hase 1970). However, due to its high toxicity this practice has been discontinued.

It has been known for a long time that the rearrangement reaction leading to the conversion of trichlorfon to DDVP occurs *in vitro* the rate being faster at higher pH (Barthel et al. 1955). However, little is known about the extent to which this conversion takes place in mammals, particularly in humans. Since trichlorfon is proposed to be administered therapeutically to patients, children in particular, in relatively large repeated doses, its conversion to DDVP might cause untoward effects of the otherwise

beneficial drug. Therefore, its conversion to DDVP and the combined effects of the two compounds should not remain unknown and uncontrolled. If DDVP is a step in the breakdown of trichlorfon, the effects of DDVP are inevitably included into data obtained by in vivo toxicological studies on trichlorfon.

Materials and Methods

Only male albino rats bred at the Institute for Medical Research and Occupational Health in Zagreb and fed on ordinary rat feed ("Pliva," Zagreb) were used in the experiments. If not otherwise stated rats were 10 to 12 weeks old and approximately 300 grams body weight.

Metrifonate (trichlorfon) was a gift from Bayer Farbenfabrik, Wuppertal, West Germany. It was in a form for human use (the purity was not stated). DDVP (91% pure) and DDT [1,1,1-trichloro 2,2-bis(p-chlorophenyl) ethane] were obtained from the World Health Organisation. TMH-4 (1,1'-trimethylene-bis-(4-formylpyridinium) and PAM-2 (pyridine-2-aldoxime methiodide both as the chloride salt were gifts of the Research Institute of "Bosnalijek" - Sarajevo. SKF-525A (N-diethylaminoethyl-3,3-diphenylpropylacetate) was a gift from late Professor Stern of Sarajevo. All other chemicals were of commercial origin.

Acute toxicity testing was done by determining LD₅₀ and ED₅₀ values after oral or intravenous administration of metrifonate or DDVP to rats of different ages. These values were calculated on the basis of 24 hour mortality rate by the method of moving averages (Thompson 1947) using the tables given by Weil (1952). The animals were carefully observed and the onset, intensity, and duration of cholinergic symptoms were recorded.

For some experiments, when a compound had to be infused or blood withdrawn, rats were anesthetized with ether, and either the jugular or a branch of portal vein were cannulated with Clay-Adams polythene cannula (PE-50 and PE-10 respectively). For slow infusion experiments, after the animals had recovered (two to three hours) metrifonate or DDVP were infused at different concentrations which were calculated so that the rate of entrance of the compound corresponded to a multiple of a single intravenous LD₅₀ value of that particular compound. The onset of cholinergic symptoms and time of death were recorded and from these values the results were calculated and expressed as numbers of respective I.V. LD₅₀ dose received until the onset of symptoms or until death.

Cholinesterase activity in plasma and brain homogenates was measured spectrophotometrically (Ellman et al. 1961). At given times animals were killed by carbon monoxide or by ether, and two

to three milliliters of blood was taken from the heart, centrifuged and plasma separated. The brain was removed, weighed and homogenized in cold saline in a concentration of 40 mg/ml. The results were expressed as percentages of the respective activity which was determined in 40 healthy rats.

The technique of the intestinal sac (Wilson and Wiseman 1954) was used for assessing metrifonate transfer through the intestinal wall. The nonverted sac of rat jejunum was filled with 0.6 ml of 0.1 M phosphate buffer pH 5.0, 7.4 or 9.0 containing 50 or 100 μ g of metrifonate. The filled intestinal sac was then placed into a buffered solution (40 ml) of hemolysed human erythrocytes, 5.0, 7.4 or 9.0. The temperature was kept at 37°C. The cholinesterase activity of the hemolysed erythrocytes was measured instantly, and at given time intervals up to 180 minutes. The enzyme activity found at "0" time was taken as 100 percent, and all other activities were expressed as percentages of that value.

Abdominal lymph was collected from large abdominal lymphatics. These were cannulated with polythene cannulae (PE-50) while rats were under ether anesthesia. After recovery, rats were dosed orally with either metrifonate or DDVP. When injected with high doses of DDVP, rats were protected with atropine (10 mg/kg) and PAM-2 (100 mg/kg) injected intramuscularly just before dosing. Lymph was collected for 30 minutes. The amount collected was divided in half and added to 4.0 ml of hemolysed human erythrocyte in phosphate buffer of pH 5.0 or 9.0. The incubation media were kept at 37°C. Cholinesterase activity was measured after five minutes of incubation and then at intervals until 60 minutes.

The TLC method (Beck and Sherman 1969) was slightly modified for the quantitative determination of metrifonate and DDVP. The two compounds were extracted with ethylacetate from blood and homogenates of brain and liver. The extract was applied directly to thin layer plates, and the plates were developed with a mixture of chloroform and acetone (1:1). The detection was carried out with AgNO_3 , and the intensity of the spots was quantified on a Model 111 fluorometer Camag (Switzerland).

Results and Discussion

Acute Toxicity in Relation to Age

The results of LD_{50} and ED_{50} (with 95% confidence limits) determined in rats of different age after oral and intravenous administration are shown in Table 31.

While LD₅₀ value for metrifonate given intravenously did not change with age, considerable differences were found after its oral administration. The youngest rats, two weeks old, were much more sensitive than adults. Surprisingly, the four weeks old were the least susceptible. The differences in DDVP treated rats were hardly noticeable. The ratios of oral and intravenous LD₅₀ values were much smaller for metrifonate than for DDVP.

In rats after the oral administration of metrifonate the onset of symptoms was much delayed as compared to DDVP. The results are shown in Table 12. Even after an intravenous injection of metrifonate there was a considerable delay in the appearance of cholinergic symptoms, while after an intravenous injection of DDVP symptoms developed within the first minute after injection.

The symptoms observed in rats given metrifonate or DDVP did not differ from those seen in rats injected with other organophosphorus compounds. They were cholinergic in nature, tremor, fasciculations and salivation dominated the clinical picture. The rats dying from high doses of either compound showed very pronounced convulsions and died of respiratory failure.

The Role of the Liver in Metrifonate Toxicity

All compounds absorbed from the gastrointestinal tract have to pass through the liver before being distributed in the body. It is well known that liver plays an essential role in many chemical processes which involve absorbed compounds. It was therefore our aim to find out whether the liver has any influence on the toxicity of metrifonate.

The effects of metrifonate infusion into the portal circulation of rats are similar to the effects when infused into the jugular vein. The tolerance of the drug until death and until the appearance of the first cholinergic symptoms was similar, regardless of the route of administration. The results are shown in Table 13. The rats infused with metrifonate at the rate of one or two single I.V. LD₅₀ doses/hour tolerated totally only slightly over one I.V. LD₅₀ dose. When DDVP was slowly infused either in the portal or in the jugular vein, the tolerance was considerably higher. Even at a rate of two I.V. LD₅₀ doses/hour, infused in the jugular vein, rats tolerated up to 17 LD₅₀ doses, the number being higher when the compound was infused into the portal vein. Also the ratio between the dose producing death and the dose producing the first noticeable symptoms was much higher for DDVP than for metrifonate by either route of administration.

It is anticipated that if the conversion of metrifonate to DDVP is assisted by the liver enzymes, their stimulation or inhibition should either increase or decrease metrifonate toxicity. The purpose of the experiments carried out in rats was to find out whether a sublethal dose of metrifonate would become lethal in rats pretreated with microsomal enzyme stimulators, or a lethal dose would become nonlethal in rats pretreated with microsomal enzyme inhibitors. The stimulating agents used were DDT or phenobarbital, and inhibitory agents were carbon disulfide or SKF-525A. The procedure was the same as described by De Matteis (1971). Altogether 14 groups, including adequate controls, totaling 324 animals were used in these experiments. DDT and CS do not seem to have influence on the toxic effects of DDVP. However, DDT, phenobarbital and SKF-525A showed a certain protective effect against the highest dose of metrifonate. The rats pretreated with CS, died after an injection of 150 or 300 mg/kg of metrifonate. It seems likely that microsomal stimulating agents decrease the acute toxicity of metrifonate by stimulating the detoxifying mechanisms. The protective effect of SKF-525A might be due to its atropiniformic action.

Although from the described experiments there was no supporting evidence that the conversion of metrifonate to DDVP is assisted by the liver, we wanted to find out more directly whether the concentration of DDVP in the blood which has just passed through the liver is higher than, equal to or lower than the concentration of DDVP in the blood coming into the liver. This was assessed by measuring the inhibitory power of the blood taken from the jugular and portal veins of rats given metrifonate or DDVP orally. Samples of blood taken either from the portal or jugular vein had a similar inhibitory effect for cholinesterase preparations after appropriate incubation *in vitro*. These results indicate that the conversion of metrifonate to another inhibitor in mammals is not influenced by the liver and therefore the conversion which takes place *in vivo* is most likely a nonenzymic process.

Brain and Plasma Cholinesterase Activity

In order to avoid any possible effect of intestinal absorption of the compounds, in these experiments we used only the intravenous route of administration. Plasma and brain cholinesterase activity was determined in rats at given times after a single injection of a sublethal dose of metrifonate (300 mg/kg) or DDVP (2.5 mg/kg).

The results are listed in Table 34. The enzyme inhibition was very pronounced shortly after dosing. The recovery of enzyme activity was also fast. Six hours after the injection the recovery was nearly complete, and on the following day inhibition was almost nonexistent. No striking differences in the

trend of recovery of either enzyme were found between rats injected with metrifonate and those injected with DDVP. Plasma cholinesterase activity regained its normal activity within a week after dosing. In rats given metrifonate brain cholinesterase activity reached its normal value only one month after the treatment.

One problem encountered in the assessment of the toxic properties of metrifonate in mammals is the aging of cholinesterases. The use of metrifonate as a drug which ought to be administered repeatedly to patients suffering from *S. haematobium* infection, bears a danger of cumulation of phosphorylated cholinesterase which cannot be reactivated, but only synthesised de novo.

The most pronounced inhibition of both brain and plasma cholinesterase was found between 15 and 30 minutes after an intravenous injection of either compound. The spontaneous enzyme recovery, however, which takes place from that time on, is more or less completed by 24 hours after dosing. Therefore, the timing 30 minutes and 24 hours was selected for measuring the extent of enzyme aging in rats dosed repeatedly with metrifonate or DDVP. Groups of six rats were injected intravenously at daily intervals with 300 mg/kg of metrifonate or 2.5 mg/kg DDVP. Brain and plasma cholinesterase activity was measured 30 minutes and 24 hours after each of five consecutive doses were injected. The results expressed as mean values for six animals are shown in Table 35.

The pattern of the enzyme inhibition was similar for the two compounds. In either case brain cholinesterase was more inhibited than plasma. The cumulation of the remaining unreacted enzyme was obvious, particularly in the brain of rats treated with metrifonate.

In a separate set of experiments rats were injected intravenously with metrifonate (300 mg/kg) or DDVP (2.5 mg/kg) and killed at given times. To measure the spontaneous reactivation of cholinesterase activity in vitro it was monitored in plasma and homogenates of brains which were taken at different times, up to 24 hours after dosing, and kept at 37°C up to 24 hrs. In rats treated with metrifonate no spontaneous reactivation occurred in homogenates of brain taken at any time after dosing and kept at 37°C. In plasma samples taken a few hours after dosing and kept under the same conditions, the recovery of enzyme activity was found to be moderate. In brain and plasma samples taken from rats by the third hour after dosing with DDVP, spontaneous reactivation continued in vitro at approximately the same rate as found in vivo.

After 2MB-4 was added to all samples taken up to six hours after dosing (at a final concentration of 3×10^{-4} M), the reactivation of cholinesterases was fast and almost complete.

All this indicates that up to approximately six hours after dosing, the spontaneous reactivation, as well as the aging of the enzymes, are completed. The lack of spontaneous reactivation in brain homogenates of rats dosed with metrifonate even soon after dosing could be understood if one takes into account the total amount of the compound injected (300 mg/kg as compared with 2.5 mg/kg of DDVP).

The observed in vivo recovery of the enzymes after administration of metrifonate agrees well with the results obtained in vitro on enzymes inhibited by DDVP (Škrinjarid-Spoljar et al. 1973).

Absorption of Metrifonate From the Gastrointestinal Tract

The pH of the gastrointestinal tract in mammals varies considerably and might have an influence on the nonenzymic rearrangement of metrifonate molecule even before its absorption. This problem has been studied in several ways.

With the intestinal sac technique it was found that regardless of the metrifonate concentration and the pH of the buffer in the sac, no inhibition of the incubation media occurred by the fifth minute of the incubation. However, after 15 minutes and at later time intervals, the extent of inhibition was very much dependent upon the dose and the pH, becoming faster with increasing concentrations and with increasing pH in the intestinal sac. The results are presented in Table 16.

The rate of inhibition by 50 µg of metrifonate at pH 9.0 was very much like that of 100 µg of metrifonate at pH 7.4. In the experiments in which the pH in the intestinal sac was 9.0 and in the incubation medium 5.0 the rate of inhibition was slightly lower than when the pH values of the media were reversed. The maximal rate of inhibition was obtained with the pH of both media was 9.0.

The question of whether or not metrifonate crosses the intestinal wall unchanged or after conversion to DDVP was also assessed in separate experiments in which inhibitory power of abdominal lymph collected from rats immediately after oral administration of metrifonate or DDVP was tested against cholinesterase preparation. As shown in Table 17, the rate of inhibition was considerably faster if the lymph from rats given metrifonate was incubated with the enzyme preparation at pH 9.0 than at pH 5.0. For comparison, rats were dosed orally with an approximately equitoxic dose of DDVP, but the inhibitory power of the collected lymph was very mild. Therefore, the dose of DDVP applied was considerably increased (300 mg/kg). The rats were protected with atropine and oximes, which in our case could not influence the results. As seen from Table 17, there was no

difference when the collected lymph was incubated with acetylcholinesterase at pH 5.0 or 7.0. The experiments of Reiner and Aldridge (1967) demonstrated that the rate of inhibition for dimethylphosphates is somewhat lower at pH 5.0 than at pH 7.0. However, a similar rate of inhibition in the case of DDVP-treated rats strongly suggests that the difference found in the metrifonate treated rats was not due to the effect of pH on inhibition. This therefore indicates that the higher the pH the faster is the conversion of metrifonate to DDVP which in turn more rapidly inhibits cholinesterase (cf. Section 9, Table 40).

Metrifonate and/or DDVP were determined by TLC method in the lymph collected from rats during the 60 minutes after oral dosing with metrifonate (300 mg/kg) or DDVP (200 mg/kg). When lymph was collected in an acid medium of pH between 2 and 3 (a drop of formic acid was added to a container before collection started) no DDVP was found in the lymph. The results are shown in Table 38. On the contrary, when lymph was not acidified and its pH was between 7 and 9, a certain amount of DDVP was found in addition to metrifonate. In the lymph of rats given DDVP the amount of DDVP recovered was similar regardless of whether the lymph was acidified or not.

These results indicate a possibility that metrifonate is transferred through the intestinal wall predominantly or entirely unchanged and that the conversion to DDVP takes place afterwards. Although the experiments described have clarified certain points in connection with the absorption of metrifonate, its pharmacokinetics remains to be elucidated.

SUMMARY

Metrifonate (trichlorfon) is a promising antiparasitic drug having a relatively low acute mammalian toxicity. This anticholinesterase compound is also in wide use as an insecticide.

The aim of the study was to study conditions which might influence its low toxicity to mammals.

In contrast to DDVP, the oral LD₅₀ for metrifonate in rats varies with age, being higher in the very young.

As with many other direct cholinesterase inhibitors, cholinergic symptoms appeared immediately after an intravenous injection of DDVP but there was a considerable delay (1 - 4 min.) in the appearance of symptoms in rats given an intravenous injection of metrifonate. Given by either route metrifonate causes inhibition of both brain and plasma cholinesterases.

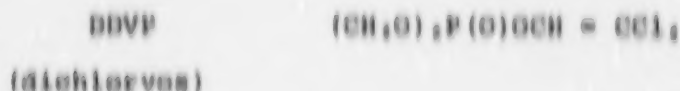
Although the rate of reactivation in vivo is fast, it is never complete, since approximately 20% of the enzyme undergoes the aging reaction. Repeated administration of metrifonate causes cumulation of the unreactivable cholinesterases.

The conversion of metrifonate to DDVP seems to be an entirely nonenzymic process, since the liver enzymes, appear to have no influence on its toxicity.

SECTION 9

REACTION IN VITRO OF METRIFONATE (TRICHLORPHON) AND DDVP (DICHLORVOS) WITH ACETYLCHOLINESTERASE AND CHOLINESTERASE

Metrifonate (trichlorfon, 0,0-dimethyl 2,2,-trichloro-1-hydroxyethyl phosphonate) and DDVP (dichlorvos, 0,0-dimethyl 2,2-dichlorovinyl phosphate) are used as pesticides, and metrifonate is also used as an antiparasitic drug. Both compounds are unstable in aqueous solutions; metrifonate rearranges into DDVP, and DDVP undergoes hydrolysis.



It is known that DDVP is an inhibitor of cholinesterases, but there are controversial views on the reaction of metrifonate with cholinesterases. The present studies were therefore undertaken to establish whether metrifonate is an inhibitor of mammalian and parasitic cholinesterases. The approach in these studies was a kinetic analysis of cholinesterase inhibition by metrifonate. In order to analyse the kinetic data it was also necessary to study the inhibition of cholinesterase by DDVP under the same conditions as those used in metrifonate studies. Further it was necessary to study the rates of spontaneous reactivation and aging of an enzyme phosphorylated by DDVP, since such data did not exist in the literature.

Methods

The activity of cholinesterases at pH 7.4 was measured by the spectrophotometric method of Ellman et al. (1961) with acetylthiocholine as substrate; the thiocholine formed upon hydrolysis of the substrate reacts with the thiol reagent DTNB forming 4-nitrothiophenol, the absorbance of which is measured

spectrophotometrically at 412 nm. The activity of cholinesterases at pH 6.0 was measured by the pH-stat method of Jensen-Holm et al. (1961) with acetylcholine as substrate; the acetic acid formed upon hydrolysis of the substrate was titrated with NaOH to maintain constant pH. The decomposition of metrifonate in buffer solutions was measured polarographically by the method of Giang and Caswell (1957). Under our experimental conditions the half-wave potential E^0 metrifonate was at -0.8 v.

Results and Discussion

The rate of inhibition of mammalian acetylcholinesterase (two species) and mammalian cholinesterase (three species) by DDVP was determined at different concentrations of DDVP and various times of inhibition (Skrinjaric-Spoljar et al. 1973; Reiner et al. 1975). All reactions followed the kinetics of a bimolecular reaction and the calculated second order rate constants of inhibition (k_i) at 25°C and 37°C are listed in Table 39. Of the five enzymes studied, human plasma cholinesterase was most inhibited by DDVP. At 37°C the rate of inhibition is faster than at 25°C, but this effect was only slightly manifested for the inhibition of horse plasma cholinesterase. This finding is in agreement with our earlier studies on the effect of temperature in which it was shown that phosphorylation and carbamylation of horse serum cholinesterase increases with temperature between 5° and 30°C, and then decreases from 30°C to 40°C (Simeon, Reiner and Vernon, 1973).

The effect of pH on inhibition of bovine erythrocyte acetylcholinesterase by DDVP is given in Table 40. At both pH 6.0 and pH 7.4 DDVP is about equally effective as inhibitor. This pH effect is consistent with our earlier finding that pH values below 8 have little effect on inhibition of acetylcholinesterase by nonionic molecules (Reiner and Aldridge 1967).

The dimethylphosphorylated enzyme, formed upon inhibition by DDVP of acetylcholinesterase and cholinesterase, undergoes two reactions which occur simultaneously: spontaneous reactivation and aging. It was shown that the rate of formation of the active enzyme EH can be calculated from the following theoretical equation (Skrinjaric-Spoljar et al. 1973):

$$[EH] = \frac{k_r}{k_r + k_{ag}} [E_0] (1 - e^{-(k_r + k_{ag})t})$$

$[E_0]$ is the concentration of the inhibited enzyme at zero time, and k_r and k_{ag} are the first order rate constants of spontaneous reactivation and aging, respectively. These constants were determined experimentally and are listed in Table 41. As shown in the table, dimethylphosphorylated human plasma

cholinesterase is very stable, and only an approximate k_i constant could be evaluated. However, human erythrocyte acetylcholinesterase reactivates spontaneously with a half-time of 51 min; the half-time of aging is 3.9 hr.

Solutions of metrifonate cause enzyme inhibition when added to mammalian acetylcholinesterase and cholinesterase. Assuming that inhibition is due only to DDVP formed from metrifonate, it is possible to calculate the rate of formation of DDVP from metrifonate. Such experiments and subsequent calculations were conducted on acetylcholinesterase from two species and cholinesterase from three species, and the results are presented in Table 42. Further, the rate of formation of DDVP from metrifonate was determined under the same experimental conditions by a direct method (polarographically), and these results are given in Table 43. The sets of values in Tables 42 and 43 are in good agreement, and such agreement is a kinetic proof that metrifonate itself is not an inhibitor of mammalian cholinesterases; inhibition by metrifonate solutions is caused by the DDVP formed from metrifonate in a nonenzymic rearrangement reaction. It has to be emphasized that in the experiments in Table 42 only 14 - 24% of metrifonate decomposed into DDVP, and the calculated k_i values are therefore subject to greater variations than those given in Table 43, which were obtained from decomposition curves where more than 50% of metrifonate decomposed.

The above conclusions are substantiated by studying the effect of pH on inhibition of mammalian cholinesterases (Table 40). The rate of enzyme inhibition by metrifonate solutions is lower at pH 6.0 than at pH 7.4. This difference is not due to the lower rate of inhibition by DDVP, but is due to the lower conversion of metrifonate into DDVP at pH 6.0.

Studies of the reaction of parasitic cholinesterases with metrifonate and DDVP are still in progress. So far only studies on the parasite Metastrongylus apri (M. apri) have been completed.

The inhibition of the cholinesterase in M. apri was measured at 25°C in 0.1 M phosphate buffer pH 7.4, and the activity was determined spectrophotometrically with acetylthiocholine and butyrylthiocholine as substrates.

DDVP is an inhibitor of the cholinesterases in M. apri. The degree of inhibition is the same irrespective of which substrate is used to measure the enzyme activity. This result provides strong evidence that acetylthiocholine and butyrylthiocholine are hydrolysed by the same cholinesterase in M. apri. The second order rate constant (k_i) of inhibition by DDVP is $7.2 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$; this is of the same order of magnitude as the k_a for the inhibition of mammalian cholinesterases.

Solutions of metrifonate inhibit the hydrolysis of acetylthiocholine and butyrylthiocholine; the degree of inhibition is the same for both substrates. From a kinetic analysis of the inhibition curves it follows that metrifonate itself is no inhibitor of the cholinesterase; inhibition is entirely due to DDVP formed from metrifonate. The kinetic analysis was the same as used in our studies of mammalian cholinesterases.

SUMMARY

Metrifonate is not an inhibitor of mammalian cholinesterases. Inhibition of mammalian cholinesterases in metrifonate solutions is due to DDVP formed from metrifonate in a nonenzymic reaction. The same conclusion applies to inhibition of the cholinesterase from the parasite Metastrongylus apri.

A comparison of three groups of results obtained in vitro (this Section) and in vivo (Section 8) supports such a conclusion.

When DDVP is injected to rats (i.v.), the onset of symptoms is immediate, while after metrifonate there is a delay of 3-4 min before the symptoms appear. The LD₅₀ for DDVP is 3-5 mg/kg and for metrifonate 450 mg/kg. If DDVP is the toxic compound, about 1% of the metrifonate has to decompose into DDVP in order to produce the toxic dose. From measurements in vitro it follows that about 3 min are required for 1% metrifonate to decompose into DDVP at 37°C. This time is in excellent agreement with the delay observed in the onset of symptoms.

Metrifonate administered to people causes inhibition of human blood cholinesterases. Plasma cholinesterase is always more inhibited than the erythrocyte acetylcholinesterase. This finding is to be expected from measurements in vitro, supposing that DDVP, and not metrifonate, is the enzyme inhibitor; the rate constant of inhibition by DDVP (measured in vitro at 37°C) is 6.6 times higher for human plasma cholinesterase than for human erythrocyte acetylcholinesterase.

The rates of spontaneous reactivation in vivo following administration of metrifonate correspond to the rates of spontaneous reactivation in vitro after inhibition by DDVP. After a single oral dose of metrifonate, human blood cholinesterases become inhibited in vivo within about half an hour; after that time the enzyme activities begin to recover. The half-time for the recovery in vivo of human plasma cholinesterase is about 180 h (calculated from mean values obtained in blood samples from 60 treated children) and the half-time for the erythrocyte acetylcholinesterase of the same group is about 100 h. The rate of spontaneous reactivation of human blood cholinesterases after inhibition in vitro by DDVP was measured at 37°C. The rate of spontaneous reactivation of

human plasma cholinesterase is slow, and only the minimum value for the rate constant could be obtained; this accounts for a half-time ≈ 81 h. Human erythrocyte acetylcholinesterase reactivates spontaneously much faster (half-time 51 min), but it also undergoes aging (half-time 233 min). It can be proved theoretically that after 300 h, 83% of the inhibited enzyme should have reactivated spontaneously.

SECTION 10

MECHANISM OF LIGAND BINDING OF ACETYLCHOLINESTERASE AND CHOLINESTERASE IN VITRO

It is known that some substrates react with cholinesterases in such way that they cause substrate inhibition (Simeon 1974 a, 1974 b). It is also known that some reversible inhibitors react with acetylcholinesterase in a way analogous to substrate inhibition (Aldridge and Reiner 1972; Reiner and Simeon 1975). It has been therefore suggested that both reactions occur at the same site, and that this site is an allosteric site of acetylcholinesterase (Aldridge and Reiner 1972). To investigate further this hypothesis, the kinetics of the enzyme-substrate and enzyme-inhibitor reaction was studied with particular emphasis on the mechanism of reversible binding of ligands to acetylcholinesterase and cholinesterase.

Materials and Methods

The enzymes were bovine erythrocyte acetylcholinesterase (Winthrop Ltd., New York, NY, USA) and horse serum cholinesterase (Sigma Chemical Comp., St. Louis, MO, USA). The substrates were acetylthiocholine iodide (AChS), phenylacetate (PhAc), benzoylcholine chloride (Bach) and acetylcholine perchlorate (ACh). The inhibitors were two organophosphorus compounds, and two carbamates. All experiments were done in 100 mM phosphate buffer pH = 7.4. The activity towards acetylthiocholine was measured by the spectrophotometric method of Ellman et al. (1961) and towards phenylacetate by the spectrophotometric method of Krupka (1966). The activity towards acetylcholine was measured by the pH-stat method of Jensen-Holm (1961). The activity of serum cholinesterase towards benzoylcholine was measured by the spectrophotometric method of Kalow et al. (1956) and the pH-stat method of Jensen-Holm (1961).

Results and Discussion

Competition between Substrates for Acetylcholinesterase and Cholinesterase

The kinetic constants for the reaction of acetylcholinesterase and cholinesterase (at 25°C) with a single substrate are

listed in Table 44. The Michaelis constant for acetylcholine and acetylcholinesterase was calculated from a plot of $[S]/v_0$ vs. $[S]$, where v_0 is the enzyme activity.

For the reaction of benzoylcholine and cholinesterase the above plot is nonlinear. The Hill equation was therefore applied, and the Michaelis constant (K_m) and the Hill coefficient (n_H) were obtained from the $K' - n_H$ analysis which was performed in order to derive the best set of K_m and n_H values which fits the experimental results. The substrate inhibition constant, K_{ss} , for benzoylcholine and cholinesterase was calculated from a plot of $1/v_0$ vs. $[S]$; this plot is linear indicating that the enzyme inhibited by the substrate itself does not hydrolyse at a measurable rate, i.e. $S = 0$ (Simeon 1974 b). The above plot for the reaction of acetylcholine and acetylcholinesterase is nonlinear indicating that S is different from zero. The K_{ss} and S values given in Table 44 were obtained from the experimental data in a way described earlier (Simeon 1974 b) by the least-squares estimation.

The competition between two substrates for their reaction with the enzyme was studied (at 25°C) so that the hydrolysis of only one substrate was measured at a time. For instance, when acetylthiocholine was the substrate and acetylcholine the inhibitor, the hydrolysis of acetylthiocholine was measured by the Ellman spectrophotometric method (1961); by this method only the formation of thiocholine, and not choline, is determined. All results were presented in such way that the substrate concentration was plotted on the abscissa, and the ratio $v [I]/(v_0 - v)$ plotted on the ordinate; v_0 is the enzyme activity when no inhibitor is present, and v is the activity at the same substrate concentration but with the inhibitor present. $[I]$ is the inhibitor concentration; the range of the inhibitor concentrations used in the experiments is given in Table 45. At each substrate concentration at least two inhibitor concentrations were tested, and each experiment was repeated 2 or 3 times. It was possible to calculate mean values because the ratio $v [I]/(v_0 - v)$ was constant at any given substrate concentration irrespective of the concentration of the inhibitor.

In the kinetic equation derived earlier (Aldridge and Reiner 1972; Reiner and Simeon 1973) the mechanism of competition between substrates and inhibitors can be identified from the numerical values of the intercepts on the abscissa and ordinate, if the results are plotted as described above. When the intercept of the line or curve with the abscissa, $K(S)$, equals the Michaelis constant, substrate and inhibitor compete at the catalytic site. When $K(S)$ equals K_{ss} , the competition takes place at the substrate inhibition site. The intercept with the ordinate, $K(I)$, corresponds to the dissociation constant between enzyme and inhibitor. In Table 45 are listed the intercepts calculated for the reactions studied. In all reactions

but one the plot of $v(I)/(v_{\infty}-v)$ vs. $[S]$ is linear. The exception is the hydrolysis of acetylthiocholine by cholinesterase with phenylacetate as inhibitor; the intercepts in that case were assessed from the initial slope of the curve. For both enzymes, the calculated $K(S)$ and $K(I)$ constants are all of the same order of magnitude as the corresponding Michaelis constants for the same compound when used as a single substrate. This is true irrespective of whether the enzyme is or is not inhibited by the single substrate. (i.e. K_m does or does not reveal itself within the studied concentration range). It also applies irrespective of whether or not the reaction of the single substrate follows the Michaelis or Hill equation.

The standard errors of the calculated constants are large (Table 45). Therefore, the differences which exist between K_m on one side, and $K(S)$ and $K(I)$ on the other side cannot be taken as meaningful in terms of reaction mechanisms. However, the values of $K(S)$ and $K(I)$ for acetylcholine, acetylthiocholine and benzoylcholine are all on the average two orders of magnitude lower than the corresponding substrate inhibition constants K_{si} . This implies that $K(S)$ and $K(I)$ do not correspond to K_{si} . It also implies that the competition between two substrates is basically a competition at the catalytic site of the enzyme. The substrate inhibition site does not show up in the kinetics of competition between substrates. This is different from the competition between the same substrates and coumarin derivatives where the substrate inhibition site was kinetically prevalent.

Effect of Temperature on Phosphorylation and Carbamylation of Acetylcholinesterase

Studies of the effect of temperature on acylation reactions have proved to be effective tools in evaluating reversible intermediates in the reaction, as shown for the acylation of human and horse serum cholinesterase (Simeon, Rainer and Vernon 1972). Following up that approach, studies were expanded to bovine erythrocyte acetylcholinesterase as enzyme. The inhibitors were: haloxon [(0,0-bis(3-chloroethyl)0-(3-chloro-4-methyl-7-coumarinyl)], phosphate, phosphostigmine methylsulfate (0,0-diethyl-3-trimethyl-ammoniumphenyl phosphate), neostigmine methylsulfate (3-trimethyl-ammonium-phenyl-N,N-dimethylcarbamate) and 3-isopropylphenyl-N-methylcarbamate.

The effect of temperature was studied at 7 different temperatures, between 5°C and 40°C, in 1.0 mM or 0.1 M phosphate buffer pH 7.4. The enzyme activity was measured with acetylthiocholine, acetylcholine or phenylacetate as substrates.

At a constant concentration of haloxon and phosphostigmine the plot $\log$ enzyme activity vs. time of inhibition was linear.

However, the slopes of lines, i.e., the first order rate constants, were not a linear function of the inhibitor concentration. This is a kinetic proof of a reversible complex between enzyme and inhibitor. The exception was the inhibition by phosphostigmine at 40°C for which a linear correlation was obtained. From a Wilkinson plot, the dissociation constant $K(1)$ of the reversible complex was calculated. For a given inhibitor, this constant is the same, irrespective of which method or substrate was used to determine the enzyme activity. The values of $K(1)$ and of the second order rate constants of phosphorylation (k_2) are given in Table 46. The increase of the rate constants of phosphorylation with temperature follows the Arrhenius equation, with an activation energy of 30 kcal mol⁻¹ for inhibition by phosphostigmine.

The inhibition of acetylcholinesterase by the two carbamate inhibitors follows the kinetics of a second order reaction with one component (the inhibitor) in excess. No reversible complex between enzyme and inhibitor is detectable from the kinetic analysis. The values of the k_2 constants are given in Table 47. The rates of carbamylation increase with increasing temperature and follow the Arrhenius equation. The activation energies for inhibition by neostigmine and 3-isopropylphenyl-N-methylcarbamate are 13 and 10 kcal mol⁻¹ respectively.

SUMMARY

The kinetics of competition of pairs of two substrates for bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and horse serum cholinesterase (acetylcholine acylhydrolase, EC 3.1.1.8) was studied (at 25°C) so that the hydrolysis of only one substrate was measured at a time. The substrates were acetylthiocholine, phenylacetate and benzoylcholine; the same compounds, and also acetylcholine, were used as competing substrates i.e. inhibitors. The substrate inhibition constants (K_{ss}) and Michaelis constants for the reaction of a single substrate were also determined. It was concluded that the substrate inhibition site in the enzyme does not show up in the competition between two substrates.

The effect of temperature (5° - 40°C) was studied on reversible and irreversible inhibition of bovine erythrocyte acetylcholinesterase by two organophosphorus compounds and two carbamates. The second order rate constants of inhibition increased with increasing temperature, and the increase followed the Arrhenius equation. From the kinetics of phosphorylation by the two organophosphorus compounds (haloxon and phosphostigmine) it was concluded that both compounds form a reversible complex with the enzyme. No reversible complex could be detected from the kinetics of carbamylation by neostigmine and 3-isopropylphenyl-N-methylcarbamate.

SECTION 11

EVALUATION OF FIELD METHODS FOR DETERMINING THE ACTIVITY OF HUMAN BLOOD CHOLINESTERASES

Absorption of organophosphorus compounds and carbamates are so far best determined by measuring the activity of human blood cholinesterases. Sometimes it is not possible to measure the activity in a laboratory, and suitable field methods are therefore required. In this report, three methods are compared and temperature conversion tables are provided for one of the methods.

Methods

The following methods were used: spectrophotometric method of Ellman et al. (1961) and two field methods, the Acholast (Hersfeld and Stumpf 1955; Flastina 1966) and tintometric (Edson 1958; Vandekar and Svetlicic 1966).

Results and Discussion

The spectrophotometric method of Ellman et al. (1961) is widely used for measuring the activity of acetylcholinesterase and cholinesterase. The procedure is simple and the results well reproducible. The method is used as a laboratory method and more recently attempts have been underway to apply it in the field. When the enzyme activity is measured under field conditions, the temperature of assay is that of its surrounding. Temperature conversion tables are therefore required to enable the conversion of activities determined at different temperatures. As no temperature conversion tables have been published for the Ellman method, the aim of our work was to provide such tables (Reiner et al. 1974) showing the effect of temperature on human blood cholinesterases.

The following experimental conditions were chosen: temperature range 10°C to 40°C, substrate concentration 1.0 mM acetylthiocholine, pH of assay 7.4, phosphate buffer 0.1 M. The above temperature range seems to cover field conditions in different countries of the world. The substrate concentration of 1.0 mM was chosen for both human whole blood and plasma cholinesterase

because it is convenient to have only one substrate concentration in routine measurements, and this concentration in routine measurements, and this concentration gives suitable activities for both preparations. At a pH of 7.4, the nonenzymic hydrolysis of 1.0 mM acetylthiocholine is slow, and no corrections for spontaneous hydrolysis are required. The phosphate buffer was chosen because the effect of temperature on the pH of that buffer is small; and the buffer can therefore be prepared at any temperature between 10°C and 40°C. The buffer concentration of 0.1 M has a good buffering capacity; the addition of whole blood or plasma will not alter the pH, also the pH will remain constant during assay.

The results are presented in Table 48. When the temperature increases from 10°C to 40°C, the activity of whole blood cholinesterase will increase 2.60 times and that of plasma cholinesterase 3.48 times.

Besides the Ellman method there are two other methods which are also widely used for determining the activity of human blood cholinesterases. These are the Achelest (Hersfeld and Stumpf 1955; Flastina 1966) and the tintometric (Edson 1958; Vandekar and Svetlicic 1966) methods. Both are field methods and they were developed to evaluate the absorption of organophosphorus compounds. When these methods are used for measuring enzyme activities in persons exposed to dimethylphosphates or carbamates their accuracy requires verification. This is because of the spontaneous reactivation of the inhibited enzyme which might give rise to inaccurate results.

The two field methods were tested under laboratory conditions and the Ellman spectrophotometric method was used as a reference method (Wilhelm et al. 1973). The inhibitors were four N-methylcarbamates. The results are presented in Tables 49 and 50. When plasma cholinesterase activity was measured by the Achelest and Ellman method, no difference was found. However, the enzyme activity in whole blood determined by the tintometric method was $\pm 11\%$ higher than when the same sample was measured by the Ellman method.

It is not always possible to measure human blood cholinesterases immediately after the sampling. When blood samples of people exposed to cholinesterase inhibitors are stored, the effect of storage upon enzyme activity should be evaluated. Human blood cholinesterases were inhibited by N-methylcarbamates and stored at different pH values, temperatures and sample dilutions. Storage conditions as shown in Table 51 were found satisfactory (Wilhelm and Reiner 1973).

The same conditions are likely to apply to other cholinesterase inhibitors because massive dilution slows down cholinesterase inhibition during storage, the low pH slows down spontaneous reactivation of the enzyme (Reiner and Aldridge 1967) and the low temperature slows down both the inhibition and spontaneous reactivation. Uninhibited human blood samples treated in the same way retained their cholinesterase activities unaltered.

Summary

Temperature conversion tables for the activities of human whole blood and plasma cholinesterases were developed for the temperature range 10°C to 40°C for the spectrophotometric method of Ellman.

Storage conditions of human blood were established for measuring the cholinesterase activities after inhibition by compounds which produce an easily reactivatable enzyme.

Two field methods, the Acholast and tintometric, were shown to compare favorably with the Ellman spectrophotometric method, which was used as the reference method.

SECTION 12

QUANTITATIVE DETERMINATION OF ORGANOPHOSPHORUS PESTICIDES BY THIN-LAYER DENSITOMETRY

For the determination of organophosphorus pesticides thin-layer chromatograms were evaluated quantitatively by direct densitometric measurements of the difference in the coloration or fluorescence of spots and layer after visualization with silver nitrate or by acetylcholinesterase inhibition (Stefanac et al. 1978).

Materials and Methods

The origin of organophosphorus standards used (Table 52) is as follows: dichlorvos 91%, WHO, Geneva, Switzerland; trichlorfon, Bayer AG, Elberfeld, West Germany; Malathion 96%, the reference standard, Cat. No. 2576, EPA, (USA); Parathion 99%, Bayer AG, Leverkusen, West Germany.

Stock solutions were prepared by weighing pure substances and dissolving them in ethanol. The same solvent was used for further dilutions.

Layer: 0.5 mm of Kieselgel G nach Stahl, Type 60, Merck, Darmstadt, West Germany. TL chromatograms were developed with the solvent system chloroform : acetone (1:1) (Beck and Sherman 1968). For the separation of malathion and parathion, whenever necessary, the solvent system acetone : benzene : n-hexane (25:10:65) was used (Grove 1972).

The solvents were purified in the usual way and freshly distilled fractions were taken daily for the detection by enzyme inhibition.

Silver nitrate (AgNO_3) reagent for detection (Beck and Sherman 1968); a solution of 2.9 g AgNO_3 in 25 ml of bidistilled water was filled up to 100 ml with acetone and left to stand 10-14 days in the refrigerator before use.

Enzyme solution (Ortlieff and Frans 1965) : a 10% aqueous solution of rat plasma diluted with Sørensen's buffer pH 8.0 to volume ratio 1:1.

Substrate solution A (Ortloff and Frans 1965) : a 0.5% solution of N-methylindoxylacetate in the solvent system acetone : water (2:3).

Substrate solution B (Mendosa and Shields 1971) : 1 mg of indophenylacetate was present in each milliliter of absolute ethanolic solution.

For densitometric measurements the Camag T-Scanner (Camag, Müttenz, Switzerland) was used. This procedure was the following: the volume applied to the TL was 10 μ l throughout. After development and evaporation of solvents, the chromatograms were sprayed with the reagent for detection. Uniformly colored spots, suitable for quantitative evaluation, appeared after the plates were warmed for 30 minutes at 135°C.

The spots were brown for dichlorvos, yellow-brown for trichlorfon, dark yellow for malathion and pale yellow for parathion. Densitometric measurements were performed with a VIS light source and primary filter ($\lambda = 460-470$ nm) only.

Detection by enzyme inhibition: the developed and dried chromatograms with dichlorvos and/or trichlorfon spots were uniformly sprayed with the enzyme solution and placed for 30 minutes in a moisture saturated atmosphere. Wet plates were then cautiously sprayed with the substrate solution A. After a few minutes dark violet spots became visible in UV light (366 nm) against the intensively fluorescent background. After spraying with the substrate solution the chromatograms were placed in a dark place and measured after 30 minutes.

Densitometric measurements were performed with a VIS light source and a primary filter ($\lambda = 360$ nm) and a secondary filter ($\lambda = 415$ nm).

Bromination (Mendosa and others 1968) : parathion and malathion were oxidized by exposing the chromatograms to bromine vapors for 5 minutes. After this time the adhering bromine was expelled by flushing the chromatograms with air for 30 minutes.

The plates with a 0.25 mm thick layer were sprayed with the enzyme solution and placed for 30 minutes in a tank saturated with water vapor.

The wet chromatograms were sprayed with substrate solution B and dried at 37°C during 20 minutes. Light yellow spots on a pink background were measured with a VIS light source using the secondary filter ($\lambda = 510$ nm) only.

Results and Discussion

The calibration curves for the quantitative determination of organophosphorus pesticides were constructed by taking the ratio of sample peak area to standard peak area as the ordinate to eliminate possible variations in experimental conditions. The peak areas were determined by the Monte Carlo method (Turina et al. 1974). The ranges of linearity and reproducibilities obtained for the studied pesticides with the procedures described are shown in Table 54.

As the conversion of trichlorfon into dichlorvos is a spontaneous process the assessment of its actual quantity at a given moment has been the objective of numerous studies. Considering the different toxicities of trichlorfon and dichlorvos on one hand, and the readiness for conversion under biological conditions on the other, the usefulness of the procedure which enables simultaneous determination of both pesticides becomes evident.

In the experiments using silver nitrate as the reagent for spot detection, no evidence was found that the conversion of trichlorfon to dichlorvos takes place in the course of analysis. The reproducibility of results, for the two compounds present simultaneously, was acceptable for the amounts of 0.1-1 µg and 1-4 µg for dichlorvos and trichlorfon respectively. However, the much higher sensitivity obtained by spot detection based on cholinesterase inhibition by organophosphorus pesticides proves that the conversion goes on continuously.

Thin-layer chromatographic assay of trichlorfon using spot detection based on enzyme inhibition yielded an intense spot with an R_f value characteristic of dichlorvos and a slightly visible spot with the R_f value of trichlorfon (Table 53). The determinations of trichlorfon performed at fixed time intervals from the start of the experiment and extrapolation to zero time make it possible to assess directly, but only approximately, the trichlorfon initially present.

The measurement of fluorescence-quenching intensity as a function of the time elapsed between the spraying with the substrate and the densitometric measurement showed reproducible values within the interval of 5-65 min. As the maximum value is reached after about 30 minutes the spots of the standard were measured in the 28th and 34th minute and those of the sample in the 30th and 32nd minute.

The bromination procedure was used for the conversion of parathion and malathion to the corresponding oxygen derivatives suitable for the detection by enzyme inhibition. It was shown by a series of experiments that the sensitivity of detection does not depend on the bromination time for malathion. However, the best results for parathion were obtained with the bromination time of 5 minutes.

The described analytical procedures under carefully defined experimental conditions were successfully applied for the determination of organophosphorus pesticides in the aqueous environment and also in biological samples for toxicological research.

SUMMARY

A method for quantitative thin-layer chromatographic analysis of organophosphorus pesticides is described. The spots visualized with silver nitrate or by enzyme inhibition were quantitatively evaluated by direct densitometric measurements of chromatograms.

The detection with silver nitrate, although neither selective nor sensitive, proved to be useful for the determination of dichlorvos and trichlorfon present simultaneously in concentrations of 0.1-1 $\mu\text{g}/10\text{ ul}$ and 1-4 $\mu\text{g}/10\text{ ul}$ respectively.

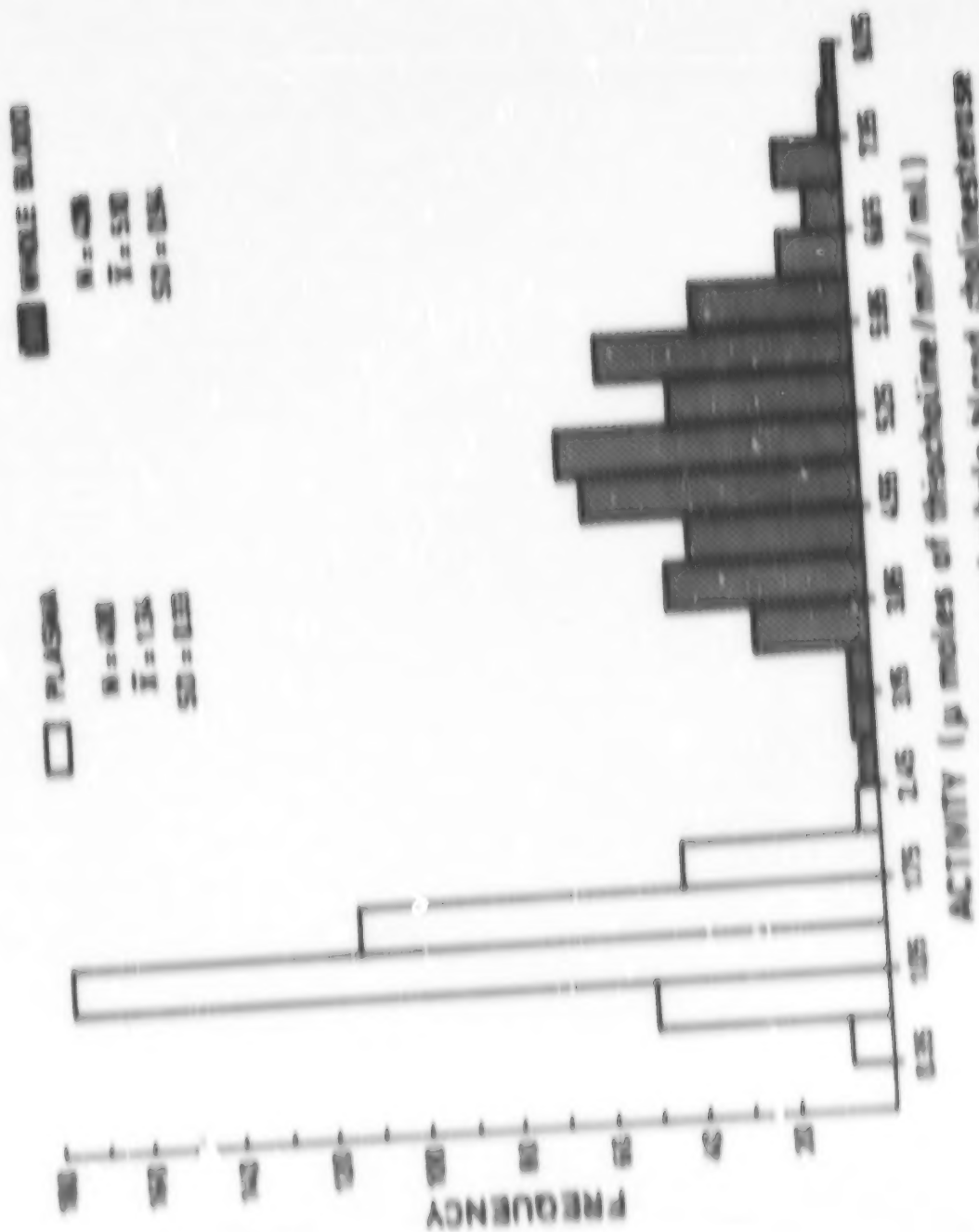


Figure 1.-Distribution of plasma and whole blood cholinesterase activity in nonexposed humans

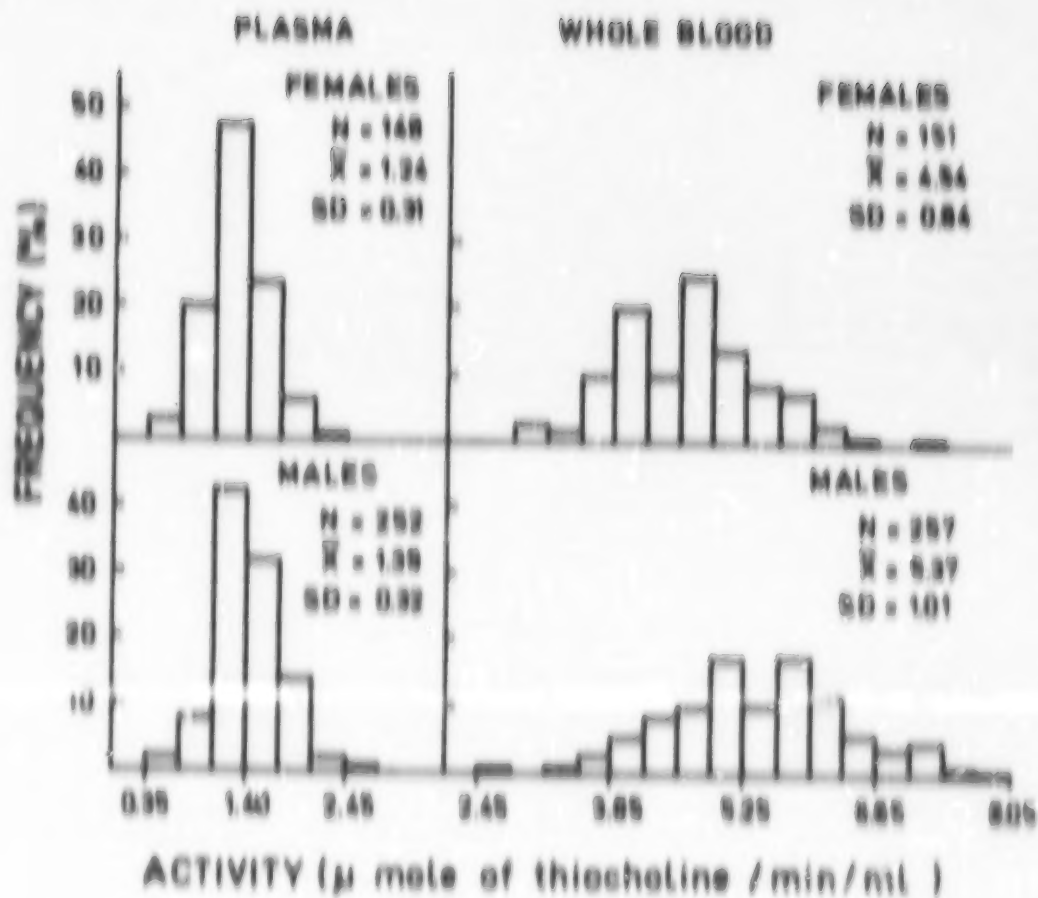


Figure 2. = Distribution of plasma and whole blood cholinesterase activity in relation to sex in nonexposed humans

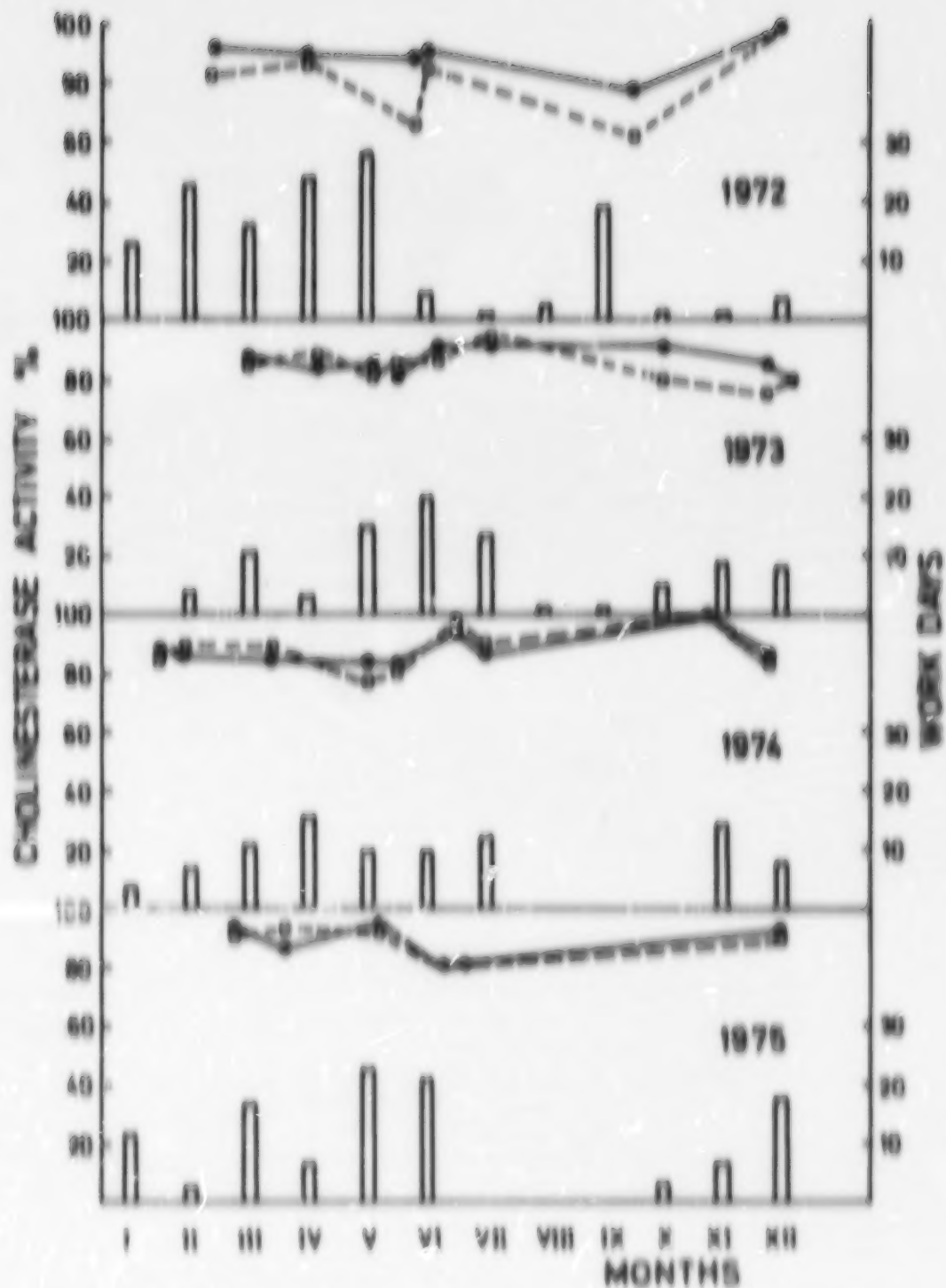
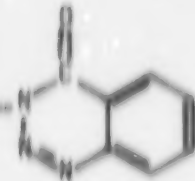
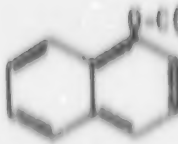
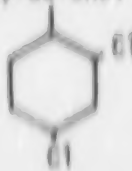


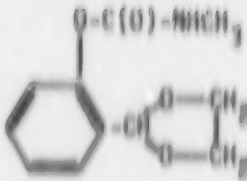
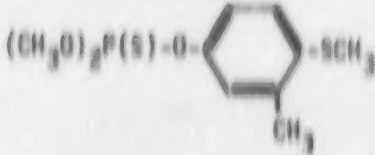
Figure 3.- Whole blood (•) and plasma (◊) cholinesterase activity in exposed workers. Number of work days is shown by means of bars (□).

TABLE 1. LIST OF COMPOUNDS HANDLED BY OCCUPATIONALLY EXPOSED WORKERS

ISO name	Synonym	Formula
AZINPHOS-ETHYL	Gusathion A	$(C_2H_5O)_2 P(S)-S-CH_2-N=N-C(=O)-C_6H_4$ 
BROMOPHOS	Nexton	$(CH_3O)_2 P(S)-O-C_6H_3(Cl)_2-Br$ 
CARBARYL	Sevin	$O-C(=O)-NHCH_3$ 
CHLORFENVINPHOS	Birlane Sapcron	$(C_2H_5O)_2 P(O)-O-C(=O)-CHCl$ 
DIAZINON	Neosudin Neosidol/ Nucidal	$(su-C_3H_7)-C_5H_3N_2-C(=O)-P(S)(C_2H_5O)_2$ 

continued

TABLE 1. (continued)

ISO name	Synonym	Formula
DICHLORVOS	DDVP Nuvon Nogos Vapona DDVP Dederap	$(\text{CH}_3\text{O})_2\text{P}(\text{O})-\text{O}-\text{CH}=\text{CCl}_2$
DIMETHOATE	Rogor Koxion Cygon Dimetate Costion MM Perfekthion	$(\text{CH}_3\text{O})_2\text{P}(\text{S})-\text{S}-\text{CH}_2-\text{CO}-\text{NHCH}_3$
DIOXACARB	Electron Famid	
FENTHION	Lebaycid Tiguven Baytex Baycid Entex Mercaptophos Qualiten	
METHIDATHION	Ultracide- Gelgy Supracide	
MOMCHLOROPHOS	Nuvacron Azodrin	$(\text{CH}_3\text{O})_2\text{P}(\text{O})-\text{O}-\text{C}(\text{CH}_3)=\text{CH}-\text{C}(\text{O})-\text{NHCH}_3$

continued

TABLE 1. (continued)

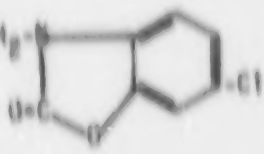
ISO name	Synonym	Formula
NALED	Dibrom	$(\text{CH}_3\text{O})_2\text{P}(\text{O})-\text{O}-\text{CHBr}-\text{CBrCl}_2$
PARATHION	Folidol Thiophos Bladen Niren Fosferno SNP	$(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{S})-\text{O}-\text{C}_6\text{H}_4-\text{NO}_2$
PHORATE	Thimate	$(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{S})-\text{S}-\text{CH}_2\text{SC}_2\text{H}_5$
PHOSALONE	Zelone	$(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{S})-\text{SCH}_2-\text{N}$ 
THIOMETON	Ekatin	$(\text{CH}_3\text{O})_2\text{P}(\text{S})-\text{S}-(\text{CH}_2)_2-\text{S}-\text{C}_2\text{H}_5$
ZINEB	Dithane Z-78 Pargatesineb	$\left[\begin{array}{c} \text{H}_2\text{C}=\text{N}-\text{C}(\text{S})\text{S} \\ \text{H}_2\text{C}=\text{N}-\text{C}(\text{S})\text{S} \end{array} \right]_n \text{Zn}$

TABLE 2. DAYS OF EXPOSURE PER ARMED (PRODUCTION OF EMULSIONS)

Year	Production (kg)	Color- Swarmings (200g)	Dichlorom (200g)	Dichlorom (400g)	NaCl (50.0g)	Non- crystalline (200g)	Total (days)
1971	18.3	37.5	0.6	26.6	-	-	74.9
1972	17.5	36.6	10.5	1.6	4.1	-	92.1
1973	72.9	2.3	7.3	1.1	0.3	8.9	92.7
1974	95.9	0	10.0	1.9	0	1.2	109.2
1975	88.0	0	5.0	2.9	0	1.0	96.9

The concentration of the active matter in the final product is given in parentheses.

TABLE 3. DAYS OF EXPOSURE PER WORKER (PRODUCTION OF SPRAYS)

Year	Dichlorvos (0.8%)	Dichlorvos (0.3%) Bromophos (0.8%)	Bromophos (0.1%)	Total (days)
1971	60	0	120	180
1972	48	0	48	96
1973	77	26	27	130
1974	72	48	30	150
1975	85	57	22	164

The concentration of the active matter in the final product is given in parentheses.

TABLE 4. DAYS OF EXPOSURE PER WORKER (PRODUCTION OF POWDERED INSECTICIDES)

Year	Zineb (68%)	Dioxacarb (2.5%)	Carbaryl (50%)	Phosalone- suspension (30%)	Total (days)
1973	9	106	85	8	208
1974	-	117	48	-	165
1975	-	96	-	108	204

The concentration of the active matter in the final product is given in parentheses.

TABLE 5. AVERAGE NUMBER OF OVERTIME WORK HOURS PER WORKER IN THE PRODUCTION OF EMULSIONS

Year	Phosalone	Chlorfenvinphos	Dimethoate	Total (hours)
1971	13.6	35.6	28.9	78.1
1972	5.0	49.8	6.1	60.9

TABLE 6. THE RELATIONSHIP BETWEEN BLOOD CHOLINESTERASE ACTIVITY AND SIGNS AND SYMPTOMS OF POISONING IN WORKERS MANUFACTURING ANTICHOLINESTERASE PESTICIDES IN ONE PLANT

Year	Inhibition of cholinesterase activity in whole blood (%)	Number of individuals	
		without complaints	with complaints and/or symptoms
1971	0 - 25	69	1
	25 - 50	10	14
	50 - 75	5	6
	75 - 100	2	3
		86	24
1972	0 - 25	99	-
	25 - 50	6	-
	50 - 75	-	1
	75 - 100	-	-
		105	1
1973	0 - 25	47	4
	25 - 50	19	1
	50 - 75	1	1
	75 - 100	-	-
		67	6
1974	0 - 25	56	-
	25 - 50	37	14
	50 - 75	9	0
	75 - 100	-	1
		102	15
1975	0 - 25	83	1
	25 - 50	71	9
	50 - 75	6	0
	75 - 100	1	1
		161	11
Total		517	67

TABLE 7. SYMPTOMS OF POISONING AND CHOLINESTERASE ACTIVITIES OF EXPOSED WORKERS

Subjects	Exposure to Compound	Duration of Exposure	Signs and symptoms recorded	Whole blood ChE activity (%)	
				while having symptoms	two weeks later
M.B.	Dimethoate Phosalone	2 months	loss of appetite, weakness	55	70
S.O.J.	"	40 days	tiredness, exhaustion, tightness in chest, cough	59	65
S.V.	"	2 months	stomach discomfort	49	69
T.Z.	"	15 days	hand-sweating, headache	56	76
J.Z.	"	7 days	vomiting, weakness, salivation, tiredness, abdominal cramps	24	40
G.R.	"	33 days	vomiting, slower pupillary reaction	37	66
R.J.	"	30 days	weakness, exhaustion	44	62
J.B.	"	21 days	weakness, headache, exhaustion	40	53
K.M.	"	15 days	tiredness, sleepiness, myosis	9	22
R.Z.	"	12 days	abdominal cramps, vomiting	21	30
C.J.	Dimethoate Phosalone, Dichlorvos	34 days	tiredness, sleepiness, headache	54	72

continued

TABLE 7. (continued)

Subjects	Exposure to Compound	Duration of Exposure	Signs and symptoms recorded	Whole blood ChE activity (%)	
				while having symptoms	two weeks later
D.B.	Dimethoate Phosalone Dichlorvos	7 days	weakness, headache, vomiting	24	42
N.Z.	Dimethoate Phosalone	3 months	weakness, headache, sweating, sickness	36.5	66
H.K.	"	3 months	headache, sickness, dizziness	62.7	62.7
K.H.	Dioxacarb Carbaryl	4 months	tightness in chest, stiff extremities	37.6	83.1
K.S.	"	4 months	dizziness, sickness, vomiting	41.6	87.2
V.J.	Phosalone	20 days	headache, sickness	61.4	61.4
B.V.	OP and car- bamate in- secticides	Continuous laboratory work	sweating, tiredness, vomiting	47.8	70.7
B.J.	"	"	weakness, vomiting, suffocation	44.4	71.1

TABLE 3. BLOOD AND PLASMA CHOLINESTERASE ACTIVITY DURING A WORKDAY*

Worker	Enzyme preparation	Blood and plasma cholinesterase activity (x)	
		measured at 6 a.m.	measured at 1 p.m.
1	blood	90	81
	plasma	72	66
2	blood	90	83
	plasma	107	100
3	blood	98	97
	plasma	90	90
4	blood	87	82
	plasma	100	100
5	blood	78	71
	plasma	82	74
6	blood	93	93
	plasma	83	81
7	blood	96	90
	plasma	92	78
8	blood	88	87
	plasma	90	90
9	blood	95	94
	plasma	99	97
10	blood	93	94
	plasma	100	98

* Measured at the beginning and at the end of a work shift in workers from the production of emulsion (exposure to phosalone).

TABLE 9. THE MEAN VALUE OF BLOOD CHOLINESTERASE ACTIVITY IN WORKERS ENGAGED IN THE FORMULATION OF THIONETON AND DICHLORVOS*

Year	Date		Mean	± SEM	N
1970	4	February	86	2.41	20
	20	March	74	3.09	22
	8	April	72	2.21	19
	20	May	62	2.67	14
	1	September	70	3.01	18
	30	October	92	2.67	14
1971	19	April	72	5.76	9
	10	June	67	6.58	12
	17	June	63	3.20	12
1972	13	April	91	3.27	10
	19	April	80	7.61	9
1973	11	July	47	6.42	12
	16	July	63	4.77	16
	23	July	66	6.87	7
1974	9	April	91	2.44	19
1975	20	February	72	3.34	22
	3	March	75	7.74	8
	10	July	68	2.98	17
	21	July	66	3.18	19
	10	November	92	1.60	24

* Expressed in percent of their preexposure values.

TABLE 10. THE MEAN VALUE OF WHOLE BLOOD AND PLASMA CHOLINESTERASE ACTIVITY IN AGRICULTURAL WORKERS*

1973	April		May	
	After 10 days of exposure		After 10 days of exposure	
	Whole blood	Plasma	Whole blood	Plasma
Mean	100	99	98	98
SEM	± 2.57	± 3.12	± 3.25	± 4.20
N	10	9	8	8

1974	April	
	After 16 days of exposure	
	Whole blood	Plasma
Mean	87	88
SEM	± 1.49	± 1.47
N	44	43

1975	April	
	After 16 days of exposure	
	Whole blood	Plasma
Mean	95	93
SEM	± 1.08	± 1.80
N	34	33

* Expressed in percent of their preexposure values.

TABLE 11. THE MEAN VALUE OF WHOLE BLOOD AND PLASMA CHOLINESTERASE ACTIVITY IN ORCHARD SPRAYMEN*

1973	27 April		26 May		2 July	
	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Plasma
Mean	100	100	93	89	86	78
SEM	± 1.13	± 3.44	± 6.36	± 3.36	± 3.62	± 7.24
N	8	8	8	8	6	6

1975	26 May		30 May	
	(after 2-3 days of exposure)		(after 2-3 days of exposure)	
	Whole blood	Plasma	Whole blood	Plasma
Mean	94	95	97	94
SEM	± 1.36	± 1.47	± 1.26	± 0.36
N	31	32	20	20

* Expressed in percent of their preexposure values.

TABLE 12. MEAN NUMBER OF LEUKOCYTES AND LYMPHOCYTES IN WORKERS EXPOSED TO ORGANOPHOSPHORUS COMPOUNDS AND IN CONTROL GROUP

Subjects (number)	Mean values			
	1966 - 1969		1970 - 1973	
	leukocytes	lymphocytes per 100 leuk.	leukocytes	lymphocytes per 100 leuk.
Exposed workers (9)	7270	35	6964	32
Control group (46)	-	-	6164	36

TABLE 13. PATHOLOGICAL FINDINGS IN HUMANS EXPOSED TO PESTICIDES.
SUMMARY OF AN EPIDEMIOLOGICAL SURVEY

SUBJECTS (NUMBER)	EXPOSURE	FINDINGS	REFERENCE
Children (324) and adults (755)	General exposure in agricultural area	Diminished visual acuity, narrowing of the peripheral visual field, myopia and occasionally astigmatism	Ishikawa, 1973
Occupational exposed (200) and control subjects (50)	Not specified	Hearing and eye problems (unspecified) significantly higher in exposed subjects	Swillick, 1971
Adult patients (51)	Not specified	Swollen disc, atrophic disc, cataract, disturbance of smooth pursuit motion of the eye	Ono, 1971
Patients "with IP poisoning" (252)	Not specified	Unspecified eye damage	Iino, 1972
School children (40,200)	General exposure to pesticides applied in agriculture	Myopia	Tanoue and Witsui, 1975
Children (4-16 yrs) (71) and students (20)	General exposure in two different areas	Reduced vision, narrowing of the vision, refraction anomaly, affected eye movement	Ishikawa, 1977a
School children (13,351)	General exposure in agricultural area	Only in 2 cases unexplained constriction of visual fields	Fukuma, 1973

Continued

TABLE 13. (continued)

SUBJECTS (NUMBER)	EXPOSURE	FINDINGS	REFERENCE
School children by region (128)	General exposure to agricultural area (240)	5-7% ocular symptoms, visual field constriction, poor vision acutization	Ichikawa, 1972b
Children (5-15 yrs) clin. cases (7)	General exposure to agricultural area (240)	Disturbed visual acuity, narrowing of visual field, astigmatism, disturbed smooth pursuit motion	Ichikawa, et al. 1972
Children (58)	UV exposed (?) to pesticides	Disturbed visual acuity, congested papillae and discolored papillae	Ichikawa, 1972
Children from nursery school (133)	General exposure - in agric. area (Tokushima)	Narrowing of visual field (in 26) disturbed sight (in 13), abnormal background (8)	Watanabe, 1972
Children (9-11 yrs) (24,000)	General exposure - two population versus agric. population	No great difference of time versus agricultural population	Iwano, 1973
Children with IP related eye disorders (22)	Not specified	Unchanged retina, narrowed vision	Takaku, 1972
Adults with IP related eye disorders (215)	Not specified	Protein proved effective for recovery of eyesight	Takaku, 1972
Agricultural workers (198) and students (227)	Organophosphates	Aberration in intracocular pressure	Daghefij, 1971

Continued

TABLE 13. (continued)

SUBJECTS (NUMBER)	EXPOSURE	FINDINGS	REFERENCE
Farmers handling pesticides (154)	Organic mercury, chlorinated hydrocarbons, organophosphates for more than 25 yrs	Constricted visual field in 25%, disturbed accommodation in 27%	Isaacs, et al., 1971
Farmers handling pesticides (56) and other agricultural workers (716)	Different pesticides	Pharyngitis, conjunctivitis	Schubert, et al., 1967
Agricultural workers handling pesticides (113)	Malathion, DDT, diazinon, carbamate	Lacrimation, photophobia, refraction anomalies, pharyngitis, increased intraocular pressure	Schubert, et al., 1971
Agricultural workers, formerly poisoned (114)	Organophosphorus compounds	Subjective loss of eyesight in 8 workers	Isaacs and Cooper, 1966
Pesticide applicators (12)	Parathion and dichlorvos 2 to 9 yrs	No abnormalities	Misaki, et al., 1975
Outpatients with signs of pesticide injury (253)	Not specified	Myopia, astigmatism, narrowing of visual field	Isaacs, et al., 1971
Occupationally poisoned workers (24)	Different pesticides 7-25 yrs	Increased intraocular pressure, glaucoma in 4.9%, constricted visual field, retinal angiopathy	Narita, et al., 1973

(Continued)

TABLE 13. (continued)

SUBJECTS (NUMBER)	EXPOSURE	FINDINGS	REFERENCE
Workers (623)	Occupational exposure up to 18 yrs	Eye abnormality in 9.8%, narrowing of visual field, dystrophic changes of iris increased dark adaptation time	Nurminen, 1975
Workers and farmers (50)	Occupational exposure (application, formulation)	Cortical wedging	Pietsch, 1972
Agricultural workers (422)	Handling DDT and trichlorophen, 2 to 10 yrs	Glaucoma in 6.5%, corneal abnormalities	Zaryn, 1973
Agricultural workers (7)	Dibutyltin	Erythema, photophobia, conjunctivitis, constricted visual field, increased intraocular pressure	Sivitskaya and Aleksandrov, 1973
Agricultural workers (77)	Tetramethylthiuram disulfide	Conjunctivitis, decreased corneal sensitivity, increased intraocular pressure	Sivitskaya, 1974
Farmers (25)	Dibutyltin (mostly) and IP compounds	Dimness of vision, conjunctival hyperemia	Raja and Tsuji, 1974
Pesticide applicators (208)	Not specified	Corneal irritation, photophobia, lacrimation, formation of corneal vessels	Ito, 1973

Continued

TABLE 13. (continued)

SUBJECTS (NUMBER)	EXPOSURE	FINDINGS	REFERENCE
Pesticide plant workers (30)	Organochlorine, mercury, OP and carbamates, 5-10 years	Conjunctival hyperemia, tearful eyes, disturbance of adaptation to dark, color vision disturbance, visual acuity troubles	Salic-Filippini et al., 1972
Agricultural workers (250)	Parathion and other OP comp., granular, aldrin, DDE	Blepharconjunctivitis, pharyngitis, dystrophy of iris, color vision impaired, narrowing of visual field, reduced dark adaptation	Claxton, 1970
Children (after OP poisoning) and adults (34)	19 children presumed to be victims of OP poisoning, others normal children and adults	Greater threshold intensity among children exposed to pesticides	Shin, et al., 1972
Pilots (17)	TEPP	No abnormalities	Isakoff et al., 1959
Adults (5)	OP compounds	Disturbed visual acuity, visual field defects, abnormal action of eyeballs	Fuji, et al., 1975

TABLE 14. SOME DATA ON THE OPTICALLY-INDICALLY EXAMINED WORKERS

Group	Number of workers examined		Age Range mean $\pm$ S.E.	Type of work
	Male	Female	Total	
A	14	17	31	Pesticide pro- duction and formulation
			22 - 50 42.2 (± 1.6)	
B	10	2	12	Pesticide applica- tion and super- vision of production
			30 - 51 42.3 (± 1.9)	
C	20	0	20	Internal transport and work in stores
			25 - 50	

TABLE 15. COMPLAINTS OF EXAMINED WORKERS CONCERNING EYE AND SIGHT

Complaint	Group		
	A	B	C
Lacrimation	11	2	0
Photophobia	6	1	0
Burning and itching	4	4	1
Frequent conjunctivitis	1	2	1
Other complaints	6	1	6
No complaints	10	4	13

TABLE 10. RESULTS OF EXTERNAL EYE EXAMINATION IN EXPOSED AND CONTROL SUBJECTS

Findings	Group		
	A	B	C
Conjunctival infection	16	0	0
Dilated episcleral vessels	12	5	3
Abnormal pupils	2	1	1
Other abnormalities	2	2	1
Normal	4	5	9

TABLE 17. CORNEAL CURVATURE IN EXPOSED AND CONTROL SUBJECTS

Clinical parameter	Eye	Statistical parameter	Group		
			A	B	C
Difference between horizontal and vertical meridian (expressed in diopters)	Right	n	29	12	20
		$\bar{X}$	0.53	1.10	0.57
		S.E.	0.08	0.242	0.10
	Left	n	20	12	26
		$\bar{X}$	0.57	1.05	0.69
		S.E.	0.07	0.246	0.13
Corneal radius (horizontal and vertically expressed in mm)	Right	n	17	-	20
		$\bar{X}$	7.91-7.80	-	7.95-7.84
		S.E.	0.07-0.08	-	0.07-0.06
	Left	n	16	-	20
		$\bar{X}$	7.86-7.81	-	7.92-7.83
		S.E.	0.08-0.08	-	0.07-0.06

TABLE 18. VISUAL ACUITY IN EXPOSED AND CONTROL SUBJECTS BEFORE AND AFTER CORRECTION

Eye	Visual acuity	Group					
		A		B		C	
		Before	After	Before	After	Before	After
Right	> 1.0	5	9	0	0	13	13
	= 1.0	10	19	8	11	2	6
	< 1.0	8	3	4	1	5	1
Left	> 1.0	5	9	1	1	13	14
	= 1.0	17	20	7	8	3	5
	< 1.0	8	1	4	3	4	1

TABLE 19. INTRAOCULAR PRESSURE IN EXPOSED AND CONTROL SUBJECTS MEASURED BY SCHIOTZ' TONOMETER AND CONVERTED TO MILLIMETERS MERCURY

Eye	Statistical parameter	Group			Significance
		A	B	C	
Right	n	20	12	17	(A:C) $p < 0.01$
	$\bar{X}$	16.24	16.16	16.79	(B:C) $p < 0.01$
	S.E.	0.67	0.97	0.61	
Left	n	27	12	17	(A:C) $p < 0.01$
	$\bar{X}$	15.67	14.92	16.40	(B:C) $p < 0.01$
	S.E.	0.69	1.06	0.54	

TABLE 20. NUMBER OF PATIENTS WITH ABNORMALITIES OF THE EYEGROUND

Clinical findings	Group		
	A	B	C
Abnormal retinal arteries or veins	3	3	3
Degenerative or chorioretinitic changes	6	0	1
Changes of the papilla	3	0	4
Other findings	1	3	0
Normal	20	6	12

TABLE 21. THE RESULTS OF REPERCUSS MEASUREMENT OF THE RIGHT EYE IN EXPOSED MONKEYS

Group		Date											
1/1	A	15	16	17	18	19	20	21	22	23	24	25	26
	B	15	16	17	18	19	20	21	22	23	24	25	26
	C	15	16	17	18	19	20	21	22	23	24	25	26
1/2	A	15	16	17	18	19	20	21	22	23	24	25	26
	B	15	16	17	18	19	20	21	22	23	24	25	26
	C	15	16	17	18	19	20	21	22	23	24	25	26
1/3	A	15	16	17	18	19	20	21	22	23	24	25	26
	B	15	16	17	18	19	20	21	22	23	24	25	26
	C	15	16	17	18	19	20	21	22	23	24	25	26
1/4	A	15	16	17	18	19	20	21	22	23	24	25	26
	B	15	16	17	18	19	20	21	22	23	24	25	26
	C	15	16	17	18	19	20	21	22	23	24	25	26

In group A, 15-20 and 21-26 and in control subjects Group C, 15-21. Each figure is a mean value of the group expressed in degrees.

TABLE 22. THE RESULTS OF TACTICAL MEASUREMENT OF THE LEFT EYE IN CONTROL SUBJECTS

Subject-Group	Group A										Group B										Group C									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1/1	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24
1/2	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24
1/3	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24
1/4	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24
1/5	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24	15	16	17	18	19	20	21	22	23	24

In Group A, 1-10 and 11-20 and in control subjects Group C, 1-10. Each figure is a mean value of the group expressed in degrees.

TABLE 23. DATA RECAPITULATION IN EXPOSED WORKERS AND CONTROL SUBJECTS

Group	Weeks																								
	1	2	3	4	5	6	7	8	9	10	11	13	15	17	19	21	23	25							
A (n=20)	5.20	4.89	4.67	4.58	4.46	4.30	4.12	3.93	3.73	3.58	3.43	3.29	3.15	2.98	2.84	2.70	2.71	2.68							
C (n=20)	5.25	4.86	4.68	4.50	4.45	4.28	4.08	3.89	3.70	3.53	3.36	3.16	2.98	2.88	2.55	2.48	2.41	2.37							
B (n=5)	5.71	4.35	4.70	4.42	4.31	4.33	4.04	3.67	3.70	3.61	3.45	3.29	3.16	2.92	2.82	2.73	2.70	2.61							

• = p<0.05; * = p<0.02; ** = p<0.01; *** = p<0.001

The results are expressed as logarithmic means of the light intensity-activation threshold.

TABLE 25. OPERATING CONDITIONS FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF TRIALKYL PHOSPHATES

Column temperature	190-195°C
Injection block temperature	225-230°C
Detector block temperature	235-245°C
Nitrogen (carrier) flow rate	30-45 ml/min
Hydrogen flow rate	35 ± 3 ml/min
Airflow rate	235 ± 10 ml/min

TABLE 26. GAS CHROMATOGRAPHIC DATA FOR TRIALKYL PHOSPHATES, ALKALI
FLAME DETECTION SYSTEMS*

Compound	Retention time (min)	Sensitivity (pg) 4:1 signal/noise
DMAP	1.1	9
DEAP	1.4	12
DMAOTP	1.8	7
DEADTP	2.3	7

* Column 4% SE-30 and 6% OV-210. Temp. 195°C; carrier flow 40 ml
N₂/min.

TABLE 27. RECOVERIES OBTAINED FOR THE CRITICAL STAGES OF THE PROCEDURE

RECOVERY (%)		New values of $[\eta]$, η_{sp}/c (ml/g)	
		DEP	DEP
EXTRACTION	Allylating reagent prepared freshly	0.5 ml added	54 (4)
		1.5 ml added	52 (4)
		2.0 ml added	54 (5)
	Allylating reagent prepared earlier	2 days	49 (4)
		3 days	41 (5)
		1 week	48 (5)
	Allylation time	10 min	51 (4)
		20 min	48 (4)
		30 min	47 (4)
	Allylation and column chromatography		45 (3)
EXTRACTION, ALLOYLATION AND COLUMN CHROMATOGRAPHY	EXTRACTION, ALLOYLATION AND COLUMN CHROMATOGRAPHY		23 (5)

TABLE 2B. AMOUNTS OF DEADTP FOUND IN URINE SAMPLES OF UNEXPOSED SUBJECTS

Subject	DEADTP (ng/ml urine)	
	Found	Recovery (%)
M.S.	< 7	19
M.S.	< 7	20
V.B.S.	< 7	32
J.B.	< 7	21
J.S.	< 7	28
B.T.	< 7	24
V.B.S.	33	14
M.S.	< 7	21
B.T.	< 7	26

TABLE 29. AMOUNTS OF DEADTP FOUND IN URINE SAMPLES AND BLOOD CHOLINESTERASE ACTIVITIES IN OCCUPATIONALLY EXPOSED WORKERS

Subject	DEADTP (ng/ml urine)		Cholinesterase activity (% of preexposure value)	
	Found	Recovery (%)	Whole blood	Plasma
S.E.	106	13	100	100
R.V.	34	19	93	96
J.K.	22	20	94	100
H.V.	43	16	100	97
I.P.	26	12	100	99
I.H.	26	13	98	90
B.J.	30	19	100	98
I.S.	36	24	73	100
S.B.	22	19	86	86
J.S.	16	22	93	99
S.V.	41	29	92	100
J.H.	<7	26	100	86
S.D.	12	26	100	100

TABLE 30. CONCENTRATIONS OF CHLORINATED HYDROCARBONS IN 147 SAMPLES OF HUMAN PLASMA OR SERUM

Compound	Concentration (ppb)		
	Mean	± SEM	(n)*
α-HCH	3.25	± 0.50	(57)
Lindane	4.09	± 0.62	(23)
Aldrin	12.2	± 3.0	(22)
p,p'-DDE	36.3	± 1.5	(147)
p,p'-DDD	11.6	± 2.7	(7)
p,p'-DDT	22.7	± 3.3	(20)

* (n) is the number of samples which contain the particular compound.

TABLE 31. ACUTE TOXICITY OF METOPROLOL AND DHP IN RATS

Compound	Age (weeks)											
	2			4			8			12		
	Route of Administration	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)	LD ₅₀ (mg/kg)
Metoprolol	Oral	175 (145-215)	1237 (-)	1670 (953-2801)	565 (-)	565 (-)	565 (-)	565 (-)	565 (-)	565 (-)	565 (-)	565 (-)
	Intravenous	-	450 (400-500)	450 (400-500)	2.74	2.38	1.12	1.12	1.12	1.12	1.12	1.12
DHP	Oral	-	85.7 (65-113)	79.3 (60-105)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)	70.7 (55-86)
	Intravenous	-	5.4 (-)	4.2 (-)	15.9	18.9	23.6	23.6	23.6	23.6	23.6	23.6

TABLE 32. MEAN TIME OF THE ONSET OF CHOLINERGIC SYMPTOMS*

Compound	Route of Administration	Age (weeks)			
		2	4	6	12
Metrifonate	Oral	16 ⁶	10.3 ³	9.6 ⁶	11.6 ⁶
	Intravenous	-	6.0 ⁰ (16)	6.6 ⁶ (16)	4.7 ⁷ (20)
DDVP	Oral	-	2.9 ² (16)	4.0 ⁰ (16)	4.9 ⁹ (16)
	Intravenous	-	<1	<1	<1

* (in minutes) after oral or intravenous administration of metrifonate or DDVP. The number of rats is given in parentheses

TABLE 33. TOLERANCE OF NEURIFUMATE AND DIMP*

Compound	Rate of infusion (I.V. $\mu\text{g}/\text{min}/\text{kg}$)	JUGULAR VEIN			POTAL VEIN		
		Initial onset of symptoms $\bar{Y}$	S.E.Y	N	Initial death Symptoms $\bar{Y}$	S.E.Y	N
Neurifumate	0.25	-	-	3.21	0.11	-	-
	0.50	0.37	0.07	2.43	0.21	0.39	0.06
	1.0	0.33	0.01	1.17	0.12	2.47	0.23
	2.0	0.44	0.05	1.25	0.08	1.25	0.12
	4.0	0.74	0.08	1.40	0.19	1.50	0.10
DIMP	0.3	0.93	0.11	2.00	0.11	1.57	0.08
	2.0	0.55	0.01	17.50	0.55	2.57	0.11
	4.0	0.72	0.05	5.24	0.13	-2.6	-31
	8.0	0.94	0.08	2.17	0.06	1.47	0.20
						1.47	0.22

* Infused into jugular or portal vein and expressed as number of intravenous $\mu\text{g}/\text{min}$ doses received until the onset of cholinergic symptoms or until death.

TABLE 34. BRAIN AND PLASMA CHOLINESTERASE ACTIVITY*

Time after dosing	Metrifonate (300 mg/kg)		DDVP (2.5 mg/kg)	
	Brain CHE	Plasma CHE	Brain CHE	Plasma CHE
30 min	1.7 (0.8)	7.0 (1.2)	14.9 (1.3)	40.6 (2.1)
60 "	6.0 (2.0)	7.0 (0.7)	29.8 (1.1)	44.3 (3.6)
90 "	12.7 (2.2)	14.3 (2.1)	36.0 (2.6)	59.2 (4.4)
120 "	13.8 (2.1)	19.4 (3.4)	40.8 (1.2)	73.7 (6.3)
180 "	34.6 (3.2)	33.3 (2.4)	58.2 (2.1)	92.7 (8.2)
6 hrs	67.6 (2.9)	73.4 (3.3)	85.1 (1.7)	94.2 (5.0)
12 "	67.9 (3.5)	73.8 (6.7)	82.6 (3.5)	99.6 (3.5)
24 "	83.8 (2.6)	94.8 (8.3)	87.4 (1.8)	102.6 (6.7)
2 days	77.6 (3.3)	93.0 (3.2)	89.5 (1.8)	106.2 (7.0)
6 "	82.6 (2.9)	95.0 (4.0)	81.9 (2.4)	101.1 (5.1)
8 "	77.4 (3.3)	101.9 (2.4)	81.9 (3.0)	88.6 (2.1)
16 "	87.5 (3.1)	"	83.7 (1.3)	"
32 "	96.2 (3.3)	"	"	"

* Percent in rats injected intravenously with a single dose of metrifonate or DDVP. Each figure is a mean value ($\pm$ S.E.X) of five to twelve animals.

TABLE 35. BRAIN AND PLASMA CHOLINESTERASE ACTIVITY*

Compound Injected	Sampling after anesth.	Number of applications				
		Time (hr)	1	2	3	4
Neostigmine 300 mg/kg i.v.	Brain	0.5 24	1.7 43.8	3.0 78.7	4.6 61.8	2.1 45.5
						2.5 42.6
Physost.	Brain	0.5 24	7.0 96.8	6.7 86.9	9.2 59.9	3.1 67.0
						4.7 72.0
DDP 2.5 mg/kg i.v.	Brain	0.5 24	14.9 67.4	20.4 92.7	26.2 76.2	18.9 64.1
						13.9 60.5
	Plasma	0.5 24	40.6 102.6	47.3 120.5	49.5 116.7	47.3 109.0
						25.4 107.9

* Percent in rats repeatedly treated with neostigmine or DDP. Each figure represents a mean value obtained in four rats.

TABLE 36. PERCENT CAL NEUTRAGE ACTIVITY AND pH*

Quantity of substrate in the sac	pH	Time of incubation (min)									
		5	15	30	45	60	90	120	180		
0	5.0	101	99	102	99	101	101	104	99	99	99
(control)	5.0	100	100	99	99	99	101	97	99	99	99
50 µg	5.0	99	96	99	97	95	87	89	79	67	67
	5.0	97	94	91	90	77	64	36	24	16	16
	5.0	92	90	90	43	15	12	11	7	5	5
	7.4	-	99	-	86	-	47	-	18	11	11
	5.0	94	81	76	99	37	16	6	5	3	3
	5.0	90	60	74	54	29	17	17	12	8	8
100 µg	5.0	97	73	53	26	11	10	6	7	6	6
	7.4	57	50	86	80	53	36	8	7	4	4
	7.4	50	88	77	51	26	17	12	5	3	3
	5.0	102	83	59	29	14	11	4	9	8	8

*In the course of incubation of the intestinal sac containing substrate in a buffered solution at different pH values. Each figure in a buffered medium at pH 5, 7.4 or 9 is a mean of four determinations.

TABLE 37. PERCENT COLTRESTENE ACTIVITY OF ABDOMINAL LYMPH AND PSP

Dose level mg/kg	pH of the medium	Incubation time (min)					
		1	5	10	15	30	45
Nefazodone (300 mg/kg p.a.)	5.0	57	77	66	54	44	31
	9.0	50	47	17*	21	12	8
DOP (45 mg/kg p.a.)	5.0	100	98	97	94	91	89
	9.0	95	96	94	93	93	88
DOP (200 mg/kg p.a.)	5.0	95	86	83	73	66	59
	9.0	98	94	88	72	62	52

* Measured in the course of incubation in a medium at different pH values of abdominal lymph collected five rats during 30 minutes after oral dosing with nefazodone or DOP.

TABLE 3B. METRIFONATE OR DDVP IN LYMPH OF RATS GIVEN METRIFONATE OR DDVP ORALLY*

Rats treated with	pH of the lymph	Compound recovered from the lymph (ug/ml)	
		Metrifonate	DDVP
Metrifonate (300 mg/kg p.o.)	7 - 8	14.4 (± 6.3)	3.8 (± 1.9)
	2 - 3	22.3 (± 2.6)	0 (0)
DDVP (7.5 mg/kg p.o.)	7 - 8	0	3.4 (± 1.3)
	2 - 3	0	2.6 (± 1.0)

* Determined by the TLC method of Beck and Sherman (1968).

TABLE 39. SECOND ORDER RATE CONSTANTS* FOR INHIBITION (k_i) OF
ACETYLCHOLINESTERASE AND CHOLINESTERASE BY DDVP

Enzyme	$10^{-4} \times k_i \text{ (M}^{-1} \text{ min}^{-1}\text{)}$	
	25°	37°
Acetylcholinesterase		
Bovine erythrocytes	2.62	-
Rat brain	6.86	16.4
Cholinesterase		
Human plasma	60.1	87.0
Horse plasma	7.77	8.78
Rat plasma	6.90	18.3

* in 0.1 M buffer pH 7.4 (from Reiner et al, 1975)

TABLE 40. EFFECT OF pH ON (a) INHIBITION OF BOVINE ERYTHROCYTE ACETYL-CHOLINESTERASE BY DDVP AND (b) ON THE RATES OF DECOMPOSITION OF METRIFONATE*

	pH 6.0	pH 7.4
(a)		
k_p (second order rate constant of inhibition by DDVP ($M^{-1} \text{ min}^{-1}$))	4.2×10^4	4.7×10^4
(b)		
k (first order rate constant of decomposition of metrifonate) (min^{-1})	$(0.73 \pm 0.12) \times 10^{-4}$	$(22.2 \pm 1.7) \times 10^{-4}$

* Values calculated from the kinetics of inhibition of bovine erythrocyte acetylcholinesterase in metrifonate solutions. Both effects were measured in 1.0 mM buffer at 37°C (from Reiner et al. 1975).

TABLE 41. FIRST-ORDER RATE CONSTANTS FOR SPONTANEOUS REACTIVATION (k_r) AND AGING (k_{ag}) OF DIMETHYLPHOSPHORYLATED ACETYLCHOLINESTERASE AND CHOLINESTERASE*

Enzyme	$10^2 (k_r \pm S.E.)$ (min^{-1})	$10^2 (k_{ag} \pm S.E.)$ (min^{-1})
Acetylcholinesterase		
Human erythrocytes	1.36 ± 0.14 (12)	0.297 ± 0.016 (20)
Bovine erythrocytes	0.92 ± 0.05 (7)	-
Rat brain	0.606 ± 0.057 (12)	0.1720 ± 0.0070 (19)
Cholinesterase		
Human plasma	≥ 0.014	-
Rat plasma	0.504 ± 0.028 (24)	approx. 0.058

* At 37°C and pH=7.4 in 0.1 M buffer. The numbers in parentheses represent the number of points from which the rate constants were calculated (from Skrinjaric-Spoljar et al, 1973)

TABLE 42. DECOMPOSITION OF METRIFONATE*

Enzyme	Metrifonate (mM)	$10^4 \times k$ (min ⁻¹)	
		25°	37°
Acetylcholinesterase			
Bovine erythrocytes	0.15	3.69	-
	0.50	2.80	-
Rat brain	0.15	6.08	28.2
	0.50	3.48	36.4
Cholinesterase			
Human plasma	0.01	7.08	41.6
	0.05	2.39	46.2
	0.10	4.17	-
Horse plasma	0.10	3.97	39.4
	0.50	2.68	30.8
	1.00	1.46	-
Rat plasma	0.15	3.13	20.0
	0.50	2.47	22.2

* In 0.1 M buffer pH 7.4. Values calculated from the kinetics of inhibition of acetylcholinesterase and cholinesterase. First order rate constants for decomposition (k) are given (from Reiner et al. 1975)

TABLE 43. DECOMPOSITION OF METRIFONATE*

Metrifonate (mM)	$10^4 \times k \text{ (min}^{-1}\text{)}$	
	25°	37°
0.150	$7.27 \pm 0.39 \text{ (33)}$	$83.1 \pm 1.2 \text{ (20)}$
15.0	$6.06 \pm 0.52 \text{ (18)}$	$37.1 \pm 1.2 \text{ (40)}$

* In 0.1 M buffer pH=7.4. The rate of decomposition was measured polarographically. First order rate constants (k) $\pm$ standard errors are given. The numbers in parentheses are the number of individual results from which k was calculated (from Reiner et al. 1975)

TABLE 44. MICHAELIS CONSTANTS (K_m) AND SUBSTRATE INHIBITION CONSTANTS (K_{si}) FOR ACETYLCHOLINESTERASE AND CHOLINESTERASE*

Substrate (mM)	K_m (mM)	K_{si} (mM)
Acetylcholinesterase		
Acetylcholine (0.01-1.0)	0.16 ± 0.01	-
Acetylcholine (1.0-100)	-	9 ($\rho=0.2$)
Acetylthiocholine	0.11	14
Phenylacetate	2.6	-
Cholinesterase		
Benzoylcholine (0.01-10)	0.4 ($n_H=0.7$)	-
Benzoylcholine (10-100)	-	56 $\pm$ 5 ($\rho=0$)
Acetylcholine	1.2 to 3.2	-
Acetylthiocholine	0.6 ($n_H=0.8$)	-
Phenylacetate	2.5	-

* n_H is the Hill coefficient. ρ is defined in the text. The range of substrate concentrations used in the experiments is given in brackets.

TABLE 4E1. INTERCEPTS ($\pm$ STANDARD ERRORS) IN THE REGRESSION $K(1)$ AND QUADRATIC $K(1)$ OF THE NUMBER AND RANGE $R_{1/2}^*$

Substrate	$K(1)$ (mM)	Inhibitor (mM)	$K(1)$ (mM)
Acetylcholinesterase			
Acetylthiocholine	0.17 ± 0.17	Acetylthiocholine (0.5-10)	0.27 ± 0.27
Acetylthiocholine	0.22 ± 0.14	Phenylacetate (2.5-15)	4.9 ± 6.1
Phenylacetate	3.8 ± 0.8	Acetylthiocholine (0.2-5)	0.25 ± 0.03
Phenylacetate	6.2 ± 1.2	Acetylthiocholine (0.5-5)	0.54 ± 0.05
Diethylacetate			
Acetylthiocholine	0.33 ± 0.17	Acetylthiocholine (5-25)	2.6 ± 0.77
Acetylthiocholine	~ 1.0	Phenylacetate (1-10)	~ 4.0
Acetylthiocholine	0.37 ± 0.55	Acetylthiocholine (0.05-1)	0.03 ± 0.04
Phenylacetate	2.5 ± 0.4	Acetylthiocholine (5-15)	3.1 ± 0.7
Acetylthiocholine	0.17 ± 0.45	Acetylthiocholine (10-50)	2.3 ± 3.3

* The range of inhibitor concentrations used in the experiments is given in brackets.

TABLE 46. EFFECT OF TEMPERATURE ON INHIBITION OF ACETYLCHOLINESTERASE BY PHOSPHOSTIGMINE AND HALOXON*

Temp. °C	Phosphostigmine			Haloxon		
	$10^{-4} k_a$ ($M^{-1}min^{-1}$)	$K(1)$ (μM)	(n)	$10^{-6} k_a$ ($M^{-1}min^{-1}$)	$K(1)$ (μM)	(n)
5.0	"	"		0.009	3.64	(20)
10	0.567	70	(5)	"	"	
11	"	"	"	0.226	4.21	(12)
16	0.944	73	(4)	"	"	
18	"	"	"	0.394	5.45	(3)
22	1.59	236	(3)	"	"	
26	"	"	"	1.25	6.42	(87)
30	3.73	124	(3)	"	"	
32	3.89	168	(3)	2.38	9.12	(3)
40	8.46	"	(7)	5.45	6.54	(7)

* k_a is the second order rate constant of phosphorylation and $K(1)$ is the dissociation constant for the enzyme-inhibitor complex (n) is the number of experiments.

TABLE 47. EFFECT OF TEMPERATURE ON INHIBITION OF ACETYL-
CHOLINESTERASE BY TWO CARBAMATES*

Inhibitor	t°C	$10^{-5} (k_a \pm \text{S.E.})$ (M ⁻¹ min ⁻¹)	(n)
3-isopropylphenyl- -N-methylcarbamate	11	2.27 ± 0.06	(5)
	25	5.35 ± 0.06	(5)
	40	10.3 ± 0.1	(6)
Neostigmine	5.6	1.59 ± 0.07	(4)
	26.6	6.71 ± 0.64	(5)
	39.5	26.1 ± 0.1	(3)

* k_a ($\pm$ standard error) is the second order rate constant of inhibition; (n) is the number of experiments.

TABLE 4B. TEMPERATURE CONVERSION TABLE FOR ACTIVITIES OF HUMAN WHOLE BLOOD AND PLASMA CHOLINESTERASES*

Temperature (°C)	Whole blood cholinesterase	Plasma cholinesterase
10	1.00	1.00
15	1.25	1.35
20	1.51	1.64
25	1.85	2.10
30	2.19	2.52
35	2.40	3.00
40	2.60	3.48

* Measured by the method of Ellman et al. (1961). Activities at 10°C were taken as 1.00 (from Rinder et al. 1974).

TABLE 49. ACTIVITY OF HUMAN WHOLE BLOOD CHOLINESTERASES AFTER INHIBITION BY DIFFERENT CARBAMATES*

Carbamate	Con- centra- tion (μ M)	Whole blood cholinesterase activity (%)	
		Tintometric method	Spectrophoto- metric method
Carbaryl	2.0	92	89
	4.0	80	76
	8.0	67	60
	16.0	54	49
Propoxur	1.0	68	65
	3.0	48	37
	9.0	36	26
Landrin	7.0	65	60
	14.0	49	44
	28.0	36	26
Promecarb	0.1	93	91
	0.4	77	72
	1.0	66	61
	4.0	30	28

* Measured by tintometric and spectrophotometric methods and expressed as a percentage of the control value (From Wilhelm et al, 1973).

TABLE 50. ACTIVITY OF HUMAN PLASMA CHOLINESTERASE AFTER INHIBITION BY DIFFERENT CARBAMATES*

Carbamate	Con- centra- tion- (μ M)	Plasma cholinesterase activity (%)	
		Acholest method	Spectrophotometric method
Propoxur	1.0	96	90
	3.0	87	86
	9.0	74	71
Landrin	7.0	67	72
	14.0	58	58
	28.0	46	46
Promecarb	0.1	87	88
	0.4	71	74
	1.0	63	60
	4.0	26	21

* Measured by Acholest and spectrophotometric methods and expressed as a percentage of the control value (from Wilhelm et al. 1973).

TABLE 61. STORAGE CONDITIONS OF HUMAN BLOOD SAMPLES FOR MEASURING CHOLINESTERASE ACTIVITY*

20 μ l blood from finger prick
↓
Diluted into 6.0 ml 20 mM buffer
pH 6.0
↓
Stored up to 4 hours at 4°C
↓
6.0 ml 0.1 M buffer pH 7.4
(containing substrate and DTNB)
added and cholinesterase acti-
vity measured by the Ellman
method

* From Wilhelm and Reiner 1973.

TABLE 52. LIST OF ORGANOPHOSPHORUS PESTICIDES DETERMINED
BY THIN-LAYER DENSITOMETRY

Name	Formula
dichlorvos (DDVP)	$(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{CH}=\text{CCl}_2$
trichlorion (metrifonate)	$(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{CH}(\text{OH})\text{CCl}_3$
malathion	$(\text{CH}_3\text{O})_2\text{P}(\text{S})\text{S}-\underset{\substack{ \\ \text{C}(\text{O})-\text{CH}_2\text{CH}_3}}{\text{CH}}-\text{CH}_2\text{C}(\text{O})-\text{OCH}_2\text{CH}_3$
parathion	$(\text{CH}_3\text{CH}_2\text{O})_2\text{P}(\text{S})\text{O}-\text{C}_6\text{H}_4(\text{p}-\text{NO}_2)$

TABLE 53. R_f VALUES OF ORGANOPHOSPHORUS PESTICIDES INVESTIGATED IN TWO SOLVENT SYSTEMS

Compound	R_f values	
	Solvent system I	Solvent system II
dichlorvos	0.52	0.30
trichlorfon	0.36	
malathion	0.62	0.53
parathion	0.62	0.64

Solvent system I, chloroform: acetone (1:1); solvent system II, benzene: acetone: n-hexane (10:25:65).

TABLE 5A. LINEARITY RANGES AND WEIGHTED VARIANCES FOR THE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF SOME DICHOROPHOSPHORUS PESTICIDES

Dichlorophosphorus pesticide	Detection of spots		
	With AgNO_3 reagent	by acetylcholineesterase inhibition	
	Range of linearity	Weighted variance	Range of linearity Weighted variance
Dichlorvos	0.1 - 0.7 μg	0.0128	0.1 - 3.0 μg 0.0632
Trichlorfon	1.0 - 5.0 μg	0.0074	100 - 300 μg -
Malathion	2.0 - 10.0 μg	0.0065	0.25-0.7 μg 0.0552
Parathion	2.0 - 10.0 μg	0.0301	0.01-4.0 μg 0.0452
Disulfoton*	0.1 - 1.0 μg	0.0200	- -
Trichlorfon*	1.0 - 4.0 μg	0.0193	- -

* Simultaneous determination.

REFERENCES

- Abraham, S. V., and Teller, J. J. 1969. Influence of various miotics on cataract formation. Br. J. Ophthalmol. 53:833-836.
- Aldridge W. N., and Reiner E. 1972. Enzyme inhibitors as substrates. Interaction of esterases with esters of organophosphorus and carbamate acids. Amsterdam: North Holland Publ. Co. XVI 326 pp., 1st Reprint, 1975.
- **Anonymous 1971. Group study on chronic OP poisoning with reference to ocular injury. Jpn. Rev. Clin. Ophthalmol. 65:387-431.
- **Araki, M., and Misuhira, V. 1973. Degenerative changes of the ciliary body of a chronically phosphoriam-inflicted beagle. J. Electron Microsc. 22:287.
- Araki, M., Otsuka, J., and Misuhira, V. 1973. Investigation of ciliary muscle of a chronically organophosphorus intoxicated beagle by means of the electron microscope and analytical electron microscope. Nippon Ganka Gakkai Sesshi (Acta Soc. Ophthalmol. Jpn.) 77:1923-1935. From: Festio. Abstr. 7(1974), no. 74-1204.
- Arthur, B. W., and Casida, J. E. 1957. Metabolism and selectivity of O,O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate and its acetyl and vinyl derivatives. J. Agric. Food. Chem. 5:186-193.
- Augustinsson, K. B. 1955. The normal variation of human blood cholinesterase activity. Acta Physiol. Scand. 35:40-57.
- Augustinsson, K. B. 1971. Determination of activity of cholinesterase. In Analysis of biogenic amines and their related enzymes, ed. D. Glick, pp. 217-273. New York: Wiley.
- Axelsson, U. 1968b. Glaucoma, miotic therapy and cataract. II. The frequency of anterior subcapsular vacuoles in glaucoma eyes treated with Para-Oxon (Mintacol). Acta Ophthalmol. 46:99-105.

- Axelsson, U. 1968a. Glaucoma, miotic therapy and cataract. I. The frequency of anterior subcapsular vacuoles in glaucoma eyes treated with echthiopate (Phospholine Iodide), pilocarpine or pilocarpine-eserine, and nonglaucomatous untreated eyes with common senile cataract. Acta Ophthalmol. 48:831-836.
- Axelsson, U. 1971. Cataracts following the use of long-acting cholinesterase inhibitors in glaucoma patients. From: The Correlation of Adverse Effects i. M. n With Observations in Animals. Proc. Eur. Soc. Study Drug Toxicity 12:199-201.
- Axelsson, U., and Holmberg, A. 1966. The frequency of cataract after miotic therapy. Acta Ophthalmol. 44:421-429.
- Barthel, W. F., Alexander, B. H., Giang, F. A., and Hall, S. A. 1955. Insecticidal phosphates obtained by a new rearrangement reaction. J. Am. Chem. Soc. 77:3424-3427.
- Beck, J., and Sherman, M. 1968. Detection by thin-layer chromatography of organophosphorus insecticides in acutely poisoned rats and chickens. Acta Pharmacol. Toxicol. 25:35-40.
- Boyd, C. L., and Bullard, T. L. 1968. Organophosphate treatments of cutaneous habronemiasis in horses. J. Am. Vet. Med. Assoc. 153:324.
- Callaway, S., Davies, D. R., and Rutland, J. P. 1951. Blood cholinesterase levels and range of personal variation in a healthy adult population. Br. Med. J. 2:812-816.
- Campbell, A. M. G. 1952. Neurological complications associated with insecticides and fungicides. Br. Med. J. 2:415-417.
- Cant, J. S., and Lewis, D. R. H. 1968. Ocular damage due to paraquat and diquat. Br. Med. J. 2:224.
- Carricaburu, P. 1971. Action du parathion sur l'electroretinogramme de la mouche Musca domestica. Crit. Rev. Acad. Sci. Paris 273:2576-2578.
- Carricaburu, P., and Lacroix, R. 1971. Effect de parathion sur l'electroretinogramme de la souris blanche. Vision Res. 11:791-796.
- Chavarria, A. P., Swartswelder, J. C., Villarejos, V. M., Ketcher, S., and Arguedas, J. 1969. Dichlorvos, an effective broad spectrum anthelmintic. Am. J. Trop. Med. Hyg. 18:907-911.

- Clark, G. 1972. Visual discrimination in chronic disulfoton poisoning. Anat. Rec. 172:291.
- Dale, W. E., J. W., Miles, and T. B. Gaines 1970. Quantitative method for determination of DDT and DDT metabolites in blood serum. J. Assoc. Off. Anal. Chem. 53:1287-1292.
- Davies, J. E. 1972. Pesticide residues in man. pp. 313-333. In Epidemiology of DDT, ed. J. E. Davies, Futura Publ. Co., Mount Kisco, N. Y.
- Davignon, L. F., (St-Pierre, J., Charest, G., and Tourangeau, F. J. 1968. A study of the chronic effects of insecticides in man. Can. Med. Assoc. J. 92:897-902.
- Davis, A., and Bailey, G. R. 1969. Metrifonate in urinary schistosomiasis. Bull. WHO 41:209-224.
- DeMatteis, J. 1971. Loss of haem in rat liver caused by the porphyrinogenic agent 2-allyl-2-isopropylacetamide. Biochem. J. 124:767-777.
- DeRoeth, A. Jr. 1966. Lens opacities in glaucoma patients on phospholine iodide therapy. Am. J. Ophthalmol. 62:619-626.
- Dubois, R. P., and Cotter, G. J. 1955. Studies on the toxicity and mechanism of action of Dipterox. AMA Arch. Ind. Health 11:53-60.
- Dugeinij, G. A. 1971. Trudovaja ekspertiza pri nabolevanijah i travmah glas u mehanizatorov sel'skogo hozjajstva i u lic imejushih kontakt s jadohimikatami. Oftalmol. Zh. 26:458-460.
- Edson, E. F. 1958. Blood tests for users of OP insecticides. World Cruph 10:1-3.
- Ellis, P. P. 1966. Systemic effects of locally applied anticholinesterase agents. Invest. Ophthalmol. 5:146-151.
- Eliman, G. E., Courtney, E. D., Andres, V., and Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95.
- Ensborg, I. F. G., DeBruin A., and Sienhuis, R. L. 1974. Health of workers exposed to a cocktail of pesticides. Int. Arch. Arbeitsmed. 32:191-201.

- Fryer, J. H., Steel, R. G. D., and Williams, H. H. 1955. Cholinesterase activity levels in normal human subjects. AMA Arch. Ind. Health 12:406-411.
- Fuji, T., Ishikawa, S., Tokumasu, K., Fawano, R., and Ozawa, H. 1975. Results of examination of equilibrating function in patients with chronic organophosphorus intoxication. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol) 69:1055-1056. From: Pestic. Abstr. 8(1975), no. 75-2633.
- Fujita, K. 1973. Cases of chemical burn of cornea by a herbicide, 11-dimethyl 4,4-bipyridinium dichloride (paraquat dichloride). Nippon Nosen Igakkai Sasho (J. Jpn. Assoc. Rural Med.) 32:154-195. From: Pestic. Abstr. 7(1974) no. 74-0336.
- Fukuto, T. R., and Metcalf, R. L. 1969. Metabolism of insecticides in plants and animals. Ann. NY Acad. Sci. 160:97-111.
- Futema, M. 1973. A screening of the visual field on 13,367 school children. Nippon Ganka Gakkai Sasho (Acta Soc. Ophthalmol. Jpn.) 77:719-730. From: Pestic. Abstr. 8(1975) no. 75-1305.
- Gage, J. C. 1955. Blood cholinesterase values in early diagnosis of excessive exposure to phosphorus insecticides. Br. Med. J. 1:1370-1378.
- Gage, J. C. 1967. The significance of blood cholinesterase activity measurements. Residue Rev. 18:159-173.
- Gaines, T. B. 1969. Acute toxicity of pesticides. Toxicol. Appl. Pharmacol. 14:513-514.
- Gehring, P. J. 1971. The cataractogenic activity of chemical agents. CRC Crit. Rev. Toxicol. 1:93-118.
- Giang, G. L., and Caswell, R. L. 1957. Polarographic determination of O,O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (Bayer L13/59). J. Agric. Food Chem. 5:753-756.
- Glasko, I. V. 1970. Dinamicheskie nabludeniya za sostoyaniem organa zreniya u lic s hronicheskoj intoksikaciej yadokhimikatami, rabotajuschih v sel'skom hoz'yajstve. Oftalmol. Zh. 25: 263-287.

- Goedde, H. W., Doernicke, A., and Atland, R. 1967. Pseudo-cholinesterases, Berlin: Springer-Verlag 177-213.
- Goeldner, H. 1960. Control of Pine Sawfly (*Diprion pini*) in forests. Hefteherbr. Bayer Pfischulka-Nachr. 13:239-244.
- Greve, P. A. 1972. Potentially hazardous substances in surface waters. I. pesticides in the River Rhine. Sci. Total Environ. 1:173-180.
- Hanyu, J., and Tsuji, T 1974. Results of investigation on the effects of agricultural chemicals, especially organophosphorus pesticides on farmers health. Koshu Eisei Joho Pul. Health Inform. 4:20. From: Pestic. Abstr. 7(1974) no. 74-1883.
- Hase, D. K. 1970. Dichlorvos - an organophosphate anthelmintic. In Topics in Medicinal Chemistry, Vol. 3, eds. J. L. Rabinowitz and R. M. Myerson, pp. 171-202. New York: Wiley.
- Hassan, A., Sayed, S. M. A. D. and Hashish, S. 1965. Metabolism of organophosphorus insecticides. VI. Mechanism of detoxification of Dipteres in the rat. Biochem. Pharmacol. 14: 1692-1694.
- Hayes, W. J., Jr. 1971. Studies on exposure during the use of anticholinesterase pesticides. Bull. WHO 44:277-288.
- Hersfeld, E., and Stumpf, G. 1955. Ein neuer Kurstest zur Bestimmung der Serumcholinesteraseaktivität. Wien Klin. Wochenschr. 67:847-876.
- Hikita, H., and Ishikawa, S. 1972. Experimental treatment of organophosphate poisoning. Inhibitory effects of GSH on corneal opacity in rats given Baytex. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:832. From: Health Aspects Pestic. Abstr. Bull. 6(1972) no. 71-0658.
- Hobbigar, F. 1963. Cholinesterases and anticholinesterase agents. In Handbuch der experimentellen Pharmakologie, Ergänzungwerk, vol. 15, p. 977. Berlin, Springer Verlag.
- Holmstedt, B. 1959. Pharmacology of organophosphorus cholinesterase inhibitors. Pharmacol. Rev. 11:567-689.
- Honne, S. 1973. Clinical symptoms and fundus findings in dogs intoxicated by organophosphorus pesticides. Rinsho Ganka (Clin. Ophthalmol.) 27:1163-1164. From: Pestic Abstr. 7(1974) no. 74-1716.

- Hornblasse, A., Adler, R. I., Vukosovich, W. M. and Gamboa, G. M. 1974. A delayed side effect of topical thiotepa. Ann. Ophthalmol., 6, 1155-1157.
- Howard, R. G., and Wilson W. S. 1972. Development of acetylcholine, choline acetyltransferase and acetylcholinesterase in rabbit corneal epithelium. Br. J. Pharmacol. 46:567-569.
- Humiston, C. G. and Wright, G. J. 1967. An automated method for determination of cholinesterase activity. Toxicol. Appl. Pharmacol. 10:467-480.
- Ichii, S., and Kimata K. 1973. Results of treatment of ocular disease due to organophosphorus pesticides for agriculture and the prognosis. Ninsho Ganka (Clin. Ophthalmol. 27:1166. From: Pestic. Abstr. 7(1974) no. 74-1612.
- Imai, H. 1974a. Toxicity of an organophosphorous pesticide (Fenthion) on the retina. Electroretinographic and biochemical study. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 78:163-172. From: author's abstract.
- *Imai, H. 1974b. Toxicity of organophosphorus pesticides (Fenthion) on the retina. Electroretinographic and biochemical study. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 78:1-10. From Pestic. Abstr. 8(1975) no. 75-2221.
- Imai, H. 1975. Toxicity of organophosphorous pesticide (Fenthion) on the retina. Electroretinographic and biochemical study. Correlative study especially on its residue actions on the retina, liver and blood cholinesterase activities and on electroretinogram. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 79:1067-1076. From: author's abstract.
- Imai, H., Miyata, M., and Ishikawa, S. 1973b. Retinal injury due to Fenthion administration. Igaku no ayumi 66:28-30. From Pestic. Abstr. 6(1973) no. 73-2699.
- Imai, H., Ono, S., Suzuki, H., Terakado, R., and Ishikawa, T. 1973a. On the electroretinogram and the electrooculogram of patients intoxicated by organophosphorus pesticides. Ninsho Ganka (Clin. Ophthalmol.) 27:1166. From: Pestic. Abstr. 7(1974) no. 74-1609.

- **Imaizumi, K. 1972. Clinical observations of ophthalmic disturbances due to agricultural chemicals. Nippon Ganka Kiyo (Folia Ophthalmol. Jpn.) 22:154-156.
- Imaizumi, K., and Atsumi, K. 1971. Eye lesions due to agricultural chemicals. Ganka (Ophthalmology) 13:71-734. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1439.
- Imaizumi, K., Atsumi, K., Mita, K., and Miyashita, H. 1971. Ophthalmologic and systemic syndromes observed in farmers with long exposure to agricultural chemicals. Nippon Ganka Kiyo (Folia Ophthalmol. Jpn.) 22:259-263. From: Health Aspects Pestic. Abstr. Bull. 5 (1972) no. 72-1693.
- Imaizumi, K., Atsumi, K., Hatano, M., Chara, Y., and Miyashita, H. 1973. Biochemical examination of eyes of rabbits intoxicated by organophosphorus pesticides. Shinsho Ganka (Clin. Ophthalmol.). 27:1163. From Pestic. Abstr. 7(1974) no. 74-1714.
- **Ishikawa, S. 1970a. Experimental study on OP ocular disease. Igaku no ayumi 73:657-658.
- **Ishikawa, S. 1970b. Treatment of OP intoxication - a case study. J. Ther. 52:1839-1848.
- **Ishikawa, S. 1970c. An outbreak of bilateral visual disturbance among school children in certain district. Jpn. J. Clin. Ophthalmol. 24:823-826.
- Ishikawa, S. 1971a. Eye injury by organic phosphorus insecticides (preliminary report). Jap. J. Ophthalmol. 15:60-68.
- *Ishikawa, S. 1971b. Eye diseases caused by organophosphates. Hokufu Saishi (Jpn. J. Public Health Nurs.) 27:58-59. In Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1898.
- Ishikawa, S. 1972a. A plan for setting up a standard of diagnosis of chronic organophosphorus poisoning due to agricultural chemicals. Ganka Shinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:817-820. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0618.
- Ishikawa, S. 1972b. Diagnostic criteria of chronic organophosphate (pesticide) poisoning. Nippon Ganka Kiyo (Folia Ophthalmol. Jpn.). 23:569-583. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-2149.

- Ishikawa, S. 1973c. Eye diseases due to organophosphate pesticides. Haigotoku (Biotech), 3(4):263-267.
- Ishikawa, S. 1973d. Pictorial presentation on organophosphate pesticide intoxication. Shingyo To Hoken (Clin. Med.) 14(7):8-14. From: Health Aspects Pestic. Abstr. Bull. 5(1973) no. 72-2184.
- **Ishikawa, S. 1973e. Experimental treatment of organophosphate poisoning. Inhibitory effects of GdCl₃ on corneal opacity in rats given Baytex. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.), 66:832-834.
- **Ishikawa, S. 1973f. Case study with OP intoxication and its diagnosis. Ophthalmology 14:78.
- Ishikawa, S. 1973a. Chronic optico-neuropathy due to environmental exposure of organophosphate pesticides (Saku disease). Clinical and experimental study. Nippon Ganka Gakkai Yasshi Jpn. (Acta Soc. Ophthalmol. Jpn.) 77:1835-1888. From: author's abstract.
- **Ishikawa, S. 1973b. Chronic organophosphate poisoning (OP). Jpn. Med. J. 1:8573.
- **Ishikawa, S. 1973c. Study on balance and eye movement in GP patients. Ophthalmic Practice, 139:5-11.
- Ishikawa, S. 1974. Optico-autonomic-peripheral neuropathy due to chronic organophosphate pesticide intoxication. In Proc. 22nd Int. Congress Ophthalmol. Paris, pp. 204-208.
- *Ishikawa, S. 1975. Neurological ophthalmology and toxicology. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 69:115-117. From: Pestic. Abstr. 8(1975) mp/ 75-10487
- Ishikawa, S. 1976. Development of myopia due to chronic organophosphate pesticides intoxication. Epidemiological and experimental study in Beagle dogs. Environ. Qual. Ref. (in press). From: author's manuscript.
- *Ishikawa, S., and Otsu, K. 1970. Study on Saku disease. Jpn. Med. J. 2425:4-15
- Ishikawa, S., and Otsu, K. 1972. Optico-neuropathy induced by environmental exposure to organophosphate pesticides. Acta Proc. Fifth Afro-Asian Congress Ophthalmol., Kyurin-aka. 434-444 (Session VI).

- Ishikawa, S., Inaba, K., Naito, M., Yamazaki, A., Fujino, T., Ota, Y., Ohta, K., and Kito, S. 1970. An obscure bi- or uni-lateral amblyopia occurring in Saku city (preliminary report). Rinsho Ganka (Clin. Ophthalmol.) 24:823-826. From: author's abstract.
- Ishikawa, S., Ueno, M., and Segawa, M. 1971. Eye diseases caused by organophosphorus compounds used in agriculture. Nanka 27:883-886. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-0084.
- *Ishikawa, S., Ueno, K. and Nasaya, S. 1972. Organophosphorus pesticide intoxication (chronic cases). No To Shinkai (Brain Nerve) 24:387-391. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1703.
- **Ishikawa, S., Imai, H., Miyata, M., Hara, T., and Nakajima, S. 1974. Experimental study due to Fenthion intoxication in rats. Development of pigmentary degeneration. The Report of the Ministry of Welfare, pp. 31-40.
- **Ishikawa, S., Mukuno, K., Miyata, M., Gono, S., and Imai, H. 1975. Treatment of chronic OP poisoning. Ophthalmic Fraction 160:481-496.
- Ishikawa, S., Gono, S., Fujii, T. 1976. Vertical gaze paresis in organophosphate pesticide intoxication. Report of five cases. Acta, Sixth Afro-Asian Congress Ophthalmol. Madras. From: author's manuscript.
- Ito, S. 1973. Chronic external ocular disturbances due to contact with pesticides. Nippon Noson Iyakkai Sasshi (J. Jpn. Assoc. Rural Med.) 23:290-291. From: Pestic. Abstr. 7(1974) no. 74-0330.
- Jensen-Holm, J. 1961. The cholinesterase activity, alone and in the presence of inhibitors, at low substrate concentrations Acta Pharmacol. Toxicol. 10:379-397.
- Kalić-Filipović, B., Đadić, S., Savić, S., Predanović, M., Arsenijević, M., Gucerić, M., and Vidaković, A. 1973. Occupational health hazards in the production and application of pesticides. Arch. Hig. Bada. Toksikol. 24:332-339.
- Kalow, W., Genest, R., and Staron, M. 1956. Kinetic studies on the hydrolysis of benzoylcholine by human serum cholinesterase. Can. J. Biochem. Physiol. 34:637-653.
- Kane, P. F. 1950. The normal variation in the interpretation of blood cholinesterase activity. Chemagro Corp. Research Dept. report no. 2662.

- Kato, S. 1972. Relation between organophosphorus pesticides and ocular anomalies. Nippon Nosen Igakkai Shassi (J. Jpn. Assoc. Rural Med.) 31:96-97. From: Health Aspects Pestic Abstr. Bull. 8(1973) no. 73-0345.
- Kato, S. 1973. Toksaj efikoj de kelkaj insektomortigiloj. Medicina Internacia Revuo 5:66-69.
- Kawai, M., and Naito, M. 1974. Experimental studies on ocular disturbances due to organophosphorus pesticides. Nippon Nosen Igakkai Shassi (J. Jpn. Assoc. Rural Med.) 33:543-544. From: Pestic. Abstr. 8(1975) no. 75-1190.
- Kawamura, A., Ogata, S., Shigeno, K., Nishimura, M., Someya, H., and Ueda, K. 1974. On the effects of long term administration of an organophosphorus pesticide to male beagles. Nippon Eiseigaku Shassi (Jpn. J. Hyg.) 29:216. From: Pestic. Abstr. 7(1974) no. 74-1699.
- Khan, M. A., Harries, W. R., and Dorward, W. J. 1973. Systemic insecticide toxicity in cattle and its prevention in Southern Alberta. Can. Vet. J. 13:129-134.
- Khan, M. A. 1973. The toxicity of Crufomate to calves exposed to warble flies (Hypoderma spp.). Res. Vet. Sci. 15:180-185.
- Kogure, M., and Imai, H. 1974. Ocular symptoms induced by organic phosphorus insecticides. J. Agr. Med. Women Assoc. 30:420-422.
- Kolmodin-Hedman, B. 1974. Exposure to Lindane and DDT and its effects on drug metabolism and serum lipoproteins. Ph.D. thesis, pp. 1-48. Stockholm.
- Komoto, M., Kikuchi, H., and Fukami, Y. 1973. Cases of apparent pesticide poisoning accompanied by myopathy. Rinsho Ganka (Jpn. J. Clin. Ophthalmol.) 27:751-755. From: Pestic. Abstr. 7(1974) no. 74-0093.
- Kono, K., Ishikawa, S., and Uga, S. 1975. Impairment of optic nerve and peripheral nerve in chronic organophosphorus intoxication. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 69:969-971. From: Pestic. Abstr. 9(1975) no. 75-2632.
- Krauthacker, R.: Methods for determination of O, O-dimethyl 2, 3, 2-trichloro-1-hydroxyethyl phosphonate (DMTHP) (in Croatian). Arch. Hig. Rada Toksikol. 26 (1975):157-169.
- Krupka, R. M. 1966. Hydrolysis of neutral substrates by acetylcholinesterase. Biochemistry 5:1983-1988.

- Kwalick, D. S. 1971. Physicians' pesticide primer. J. Med. Soc. MJ. 60, 375-377. (summary) Pestic. Abstr. no. 73-0086.
- Laties, A. M. 1969. Localization in cornea and lens of topically applied irreversible cholinesterase inhibitors. Am. J. Ophthalmol. 68:848.
- Lehner, P. N. 1973. Failure of Dieldrin to affect color vision in juvenile mallard ducks. Arch. Environ. Contam. Toxicol. 1:303-311.
- Lorenz, W., Henglein, A., and Schrader, G. 1955. The new insecticide O,O-dimethyl, 2,2,2-trichloro-1-hydroxyethyl phosphonate. J. Am. Chem. Soc. 77:2884-2886.
- Maebashi, H., Nishimura, M., Ueda, R., Ogata, S., and Kawamura, A. 1973. Experimental chronic intoxication by organophosphorus insecticides on beagles. Sonyo Igaku (Jpn. J. Ind. Health) 14:411. From: Health Aspects Pestic. Abstr. Bull. 5(1973) no. 72-2466.
- *Matsushima, S. 1973. Clinical aspects of chronic poisoning by organophosphorus pesticides. Haigotoku (Biotechnical) 3:358-363. From: Health Aspects Pestic. Abstr. Bull. 5(1973) no. 72-1206.
- Matsushima, S., Abe, S., Kurosawa, K., Yanagisawa, T., Sasaki, K., Yamazaki, H., and Shimazaki, K. 1973. Experimental study on subacute toxicity of fenitrothion. Nippon Hosen Igakkai Saishi (J. Jpn. Assoc. Rural Med.) 21:310-311. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0397.
- Matsushima, S., Abe, S., and Shimazaki, K. 1973. Experimental studies on subacute toxicity of dichlorvos to monkey and rabbit. Nippon Hosen Igakkai Saishi (J. Jpn. Assoc. Rural Med.) 22:396-397. From: Pestic. Abstr. 7(1974) no. 74-0805.
- Medvedovskaya, I. P. 1967. K voprosu o sostojani organa zrenija u kolchosnikov razlichnogo proizvodstvennogo profilja. Gig. Tr. Prof. Sabul. 11(4):37-39.
- Medvedovskaya, I. P. and Golenko, V. S. 1971. Sostojaniye organa zrenija u rabotnits krupnoblachnikh plencenikh toplite. Gig. Tr. Prof. Sabul. 15(5):32-35.
- Mendosa, C. S., and Shielde, J. B. 1971. Specificity and sensitivity to organophosphorus and carbamate pesticides: Factors affecting determination by thin-layer chromatography. J. Assoc. Off. Anal. Chem. 54:507-512.

- Mendosa, C. E., Walce, P. J., McLeod, H. A., and McKinley, W. P. 1968. Enzymatic detection of ten organophosphorus pesticides and carbaryl on thin-layer chromatograms: An evaluation of indoxyl and 1-naphthyl acetates as substrates of esterases. Analyst 93:14-18.
- Netcalf, R. L., Fukuto, T. R., and March, R. H. 1959. Toxic action of Dipterox and DDVP to the house fly. J. Econ. Entomol. 52:44-49.
- Nichon, J. Jr., and Kinoshita, J. H. 1968. Experimental miotic cataract. II. Permeability, cation transport and intermediary metabolism. Arch. Ophthalmol. 79:611.
- **Mizumi, J., Matsushita, T., and Tomio, T. 1972. Results of health investigations on persons who have engaged in application of low-toxicity organophosphorus pesticides. Sanjya Isaku (Jpn. J. Ind. Health) 14:410-411. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-2369.
- Mizumi, J., Matsushita, T., Tomio, T., and Arimatsu, Y. 1975. Health survey of pest control operators exposed to organophosphorus pesticides Kumamoto Isakui Sasshi (J. Kumamoto Med. Soc.) 49:175-184. From: Pestic. Abstr. 9(1975) no. 75-2381.
- Mitani, Y. 1972. Report of children with eye disease due to organophosphate pesticides in Tokushima Prefecture. Ganka Rinsho Iho (Jpn. Soc. Clin. Ophthalmol.) 26:822-824. From: Health Aspects Pestic. Abstr. Bull. 5(1973) no. 73-8620.
- Miyata, M., Imai, H., and Ishikawa, S. 1973. Electroretinographic study of the rat after Fenthion intoxication. Jpn. J. Ophthalmol. 17:335-343.
- Miyata, M., Imai, H., and Ishikawa, S. 1974. Characteristics and localization of cholinesterase within the retina (Effect of organophosphorus compound: Fenthion). Folia Ophthalmol. Jpn. 25:89-91. From: author's abstract.
- **Miyawaki, K. 1973. On the results of investigation on injuries due to pesticide in Waguchi Misha Town). Shikoku Eiseigaku Sasshi (J. Shikoku Public Health Soc.) 18:21-22.
- Mukuno, S., and Imai, H. 1973. A study on extracellular muscles of bees intoxicated chronically by an organophosphorus compound - histochemical and electron microscopic observation). Nippon Ganka Gakkai (Acta Soc. Ophthalmol. Jpn.) 7(1974) no. 74-8916.

- Mukuno, K., and Ishikawa, S. 1975. On toxic ocular diseases, especially the mode of toxic action on the visual system). No To Shinkai (Brain and Nerve) 27:809-820. From: Pestic. Abstr. 8(1975) no. 75-2700.
- Mukuno, K., Ishikawa, S., and Uga, S. 1973. An electron microscopic study of the extracellular muscles, optic nerve and sural nerve in experimental chronic organophosphate intoxication. J. Clin Electron Microscopy 6:254-255.
- **Mukuno, K., Ishikawa, S., and Uga, S. 1975. Study on sural nerve on OP patients). Jpn. Rev. Clin. Ophthalmol. 69:969-971.
- Müller, G. 1961. Problems of protecting maize crops in El Salvador. Hefchenbr. Bayer Pflschutz-Nachr. 14:33-49.
- Naito, M., Kawai, M., and Ueda, K. (1973a). Ocular disturbances due to organophosphorus pesticides for agriculture, Part 1. Quantitative and qualitative subchronic experiments on rats orally administered a high dose rate, LD₅₀. Nippon Ganka (Clin. Ophthalmol.) 27:1164. From: Pestic. Abstr. 7(1974) no. 74-1716.
- Naito, M., Kawai, M., and Ueda, K. (1973b). Ocular disturbances due to organophosphorus pesticides for agriculture, Part 2. Chronic experiments on rabbits sprayed with low concentrations for a long time. Nippon Ganka (Clin. Ophthalmol.) 27:1164. From: Pestic. Abstr. 7(1974) no. 74-1717.
- Nakamura, R., Kusuki, T., Mori, S., Kanemitsu, J., Kakimoto, S., Takano, T., Tsuda, T., and Kishikawa, J. 1971. Myopic tendency induced by agricultural chemicals and its treatment. Nippon Ganka Kiyo (Folia Ophthalmol. Jpn.) 22:584-590. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1444.
- Nishimura, M., Kaneko, Y., Sakama, H., Tomita, M., Yoneya, H., Ueda, K., Ogata, S., and Kawamura, A. 1974. Experiments on the toxicity of organophosphorus pesticides to beagles. Sanyo Igaku (Ind. Med.) 16:362. From: Pestic. Abstr. 8(1975) no. 75-8042.
- Nuritdinova, F. N. 1973. The hydrodynamics of the eyes in chronic pesticide poisonings). Med. Zh. Uzb. 4:27-30. From: Pestic. Abstr. 7(1974) no. 74-0320.
- Nuritdinova, F. N. 1975. O toksicheskom dejstvií fosfor-organicheskikh pesticidov na organ zreniya. Vestn. Oftalmol. 1:75-76.

- Ogata, S. 1972a. Effect of organophosphorus pesticides in beagles in chronic toxicity studies (Report I). Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 76:1143-1150. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0679.
- *Ogata, S. (1972b). Chronic toxicity studies of organophosphate in beagle dogs. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 76(Suppl. 1):185. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1730.
- Ohba, K., Mishima, S., and Ohdo, K. 1972. Visual field anomalies in Japanese juveniles from the vicinity of Saku, Nagano Prefecture, Japan. Ganka Binsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:835-837. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0823.
- Ohishi, S. 1972. High incidence of blindness of cattle in Ehime Prefecture: Discussion of casual relationship to pesticide poisoning. Nippon Seigai Igakukai Kaishi (J. Jpn. Accident Med. Assoc.) 20:191-194. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1695.
- Ohto, K. 1971. Eye diseases induced by organic phosphorus insecticides, adult case. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 75:1944-1951. From: Author's abstract.
- Ohto, K. 1972. Diagnosis and treatment of eye diseases caused by organophosphorus pesticides. Ganka (Ophthalmology) 14:17-26. From Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-0773.
- Ohto, K. 1974. Long term followup study of chronic organophosphate pesticides intoxication (Saku Disease) with special reference to retinal pigmentary degeneration. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 78:37-45. From: Pestic. Abstr. 8(1975) no. 75-2130.
- Osishi, S. 1974. Industrial intoxication and workers eye. Bodo Eisei (Ind. Hyg.) 15:50-52. From: Pestic. Abstr. 7(1974) no. 74-1618.
- Ooka, E., Komoto, M., Morikawa, S., Kikuchi, N., Arimoto, H., Gekka, M., Fukami, Y., Tanahashi, Y., and Ito, K. 1973. Eye disorders in rats experimentally poisoned with organophosphorus compounds. Nippon Ganka Gakkai Sasehi (Acta Soc. Ophthalmol. Jpn.) 77:1236-1238. From: Pestic. Abstr. 7(1974) no. 74-0938.

- *Ooka, R., Kimoto, M., Morikawa, S., Kikuchi, N., Arimoto, H., Tsukihana, R., Fukami, Y., Tanahashi, Y., and Ito, K. 1973. Ocular disturbances on experimentally poisoned rats by an organophosphorus pesticide. Nippon Ganka Gakkai Sesshi (Acta Soc. Ophthalmol. Jpn.) 77:39-40. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-2497.
- Oono, S., Imai, H., and Ishikawa, S. 1973. Two cases with typical chronic OP poisoning. (Jpn. J. Clin. Ophthalmol. 27:731-736. From: author's abstract.
- **Oota, R. 1973. Ocular disturbances on experimentally poisoned rats by an organophosphorus pesticide. Nippon Ganka Gakkai Sesshi (Acta Soc. Ophthalmol. Jpn.) 77:39-40.
- Ortloff, R. and Frans, P. 1965. Zwei neue Methoden der biochemischen Lokalisierung von phosphorhaltigen Insektiziden auf Dünnschichtchromatogrammen. Z. Chem. 5:388-389.
- **Oshiba, K. 1970. Interaction between toxicant and nutrition. II. Relationship between concentrations of BHC in diet and deposition of the chemical in animal tissues. Shokuhin Rinsigaku Sesshi (J. Food Hyg. Soc. Jpn.) 11:448-449.
- *Oto, K. 1973. Treatment of ocular disturbances due to organophosphorus pesticides used in agriculture. Rinsho Ganka (Clin. Ophthalmol.) 27:1166. From: Pestic. Abstr. 7(1974) no. 74-1887.
- Otsuka, J. 1971. A followup report on eye damage caused by organophosphate pesticides. Ganka (Ophthalmology) 13:715. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1531.
- *Otsuka, J., and Tokoro, T. 1972. Study on a standard of diagnosis of eye diseases due to organophosphate pesticides from the viewpoint of eye refraction. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:825. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0621.
- Otsuka, J., Tokoro, T., Suzuki, K., and Suzuki, H. 1972. Development of myopia induced by organic phosphorus pesticides. Acta. Proc. Fifth Afro-Asian Congress of Ophthalmol. Kyushu-oka, pp. 445-449.
- Ozawa, T., Takase, M., and Manabe, Y. 1972. Three cases of ophthalmological disorders possibly induced by organic phosphorus insecticides. Teishin Igaku (Med. J. Commun) 24:27-29. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-1252.

- Osawa, H., Usui, H., Hirasawa, Y., Obayashi, M., and Ishikawa, S. 1975. Equilibrating function and eyeball motion determined by a newly developed gravicorder. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol.). 59:1057-1059. From: Pestic Abstr. 8(1975) no. 75-2634.
- Paccagnella, B., Ghesso, F., Prati, L., Fedrassoni, U., and Ballini, G. 1971. Epidemiological study of longterm effects of pesticides on human health; Bull. WHO 45:181-199.
- Paterson, C. A. 1971. Effects of drugs on the lens. Int. Ophthalmol. Clin. 11:63-97.
- Pietsch, R. L., Bobo, C. B., Finkles, J. F., and Valletton, W. W. 1972. Lens opacities and organophosphate cholinesterase-inhibiting agents; Am. J. Ophthalmol. 73:236-242.
- Plesina, R. 1966. Our experience in applying the Acholust method for determination of human plasma cholinesterase. Arch. Hig. Bada Toksikol. 17:291-301.
- Plestina, R., Davis, A., and Bailey, D. R., 1972. Effect of metrifonate on blood cholinesterases in children during the treatment of schistosomiasis. Bull. WHO 46:747-759.
- Reiner, E. (Editor): "Cholinesterases and cholinergic receptors"; Prog. Int. Med., Split 1975; Croatian Chemical Acta; Zagreb (1975); Nil + 344 pp.
- Reiner, E. 1971. Spontaneous reactivation of phosphorylated and carbamylated cholinesterases. Bull. WHO 44:109-112.
- Reiner, E., and Aldridge, W. N. 1967. Effect of pH on inhibition and spontaneous reactivation of acetylcholinesterase treated with esters of phosphorus acids and of carbamic acids. Biochem. J. 105:171-179.
- Reiner, E., Buntić, A., Trdak, M., and Simeon, V. 1974. Effect of temperature on the activity of human blood cholinesterases. Arch. Toxicol. 32:347-350.
- Reiner, E., Krauthacker, B., Simeon, V., and Skrinjaric-Spoljar, M. 1975. Mechanism of inhibition *in vitro* of mammalian acetylcholinesterase and cholinesterase in solutions of O, O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (Trichlorophen). Biochem. Pharmacol. 24:717-722.
- Reiner, E., and Simeon, V. 1975. The Proceedings of the International Meeting on Cholinesterases and Cholinergic Receptors. Croatian Chem. Acta 47:321-331.

- Robbins, W. E., Hopkins, T. L., and Eddy, G. W. 1956. La métabolisation du "Dayer L-13/59" par la vache, contrôlée à l'aide d'un produit marqué d'un ^{14} P radioactif. J. Econ. Entomol. 49:601-606.
- Rubin, L. S., and Goldberg, M. N. 1957. Effect of Sarin on dark adaptation in man: Threshold changes. J. Appl. Physiol. 11:439-444.
- Rubin, L. S., and Goldberg, M. N. 1958. Effect of tertiary and quaternary atropine salts on absolute scotopic threshold changes produced by an anticholinesterase (Sarin). J. Appl. Physiol. 12:305-310.
- Rubin, L. S., Krop, S., and Goldberg, M. N. 1957. Effect of Sarin on dark adaptation in man: Mechanism of action. J. Appl. Physiol. 11:445-449.
- Saito, M., and Kubo, H. 1972. Report of children with eye diseases due to organophosphate pesticides from a biochemical point of view. Ganka Shinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:828-883. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0622.
- Sandifer, S. H., Keil, J. E., Finkles, J. F., and Gadsden, R. H. 1972. Pesticide effects on occupationally exposed workers. Ind. Med. 41:9-12.
- Shaffer, R. N., and Hetherington, J. 1966. Anticholinesterase drugs and cataracts Am. J. Ophthalmol. 62:613-618.
- Shafik, M. T., Moseman, R. F., and Mann, J. B., eds. 1974. Analysis of pesticides residues in human and environmental samples. A compilation of methods selected for use in pesticide monitoring programs. RTP U.S. Environmental Protection Agency.
- **Shikano, S. 1972. Governmental report of school children with bilateral visual disturbance. Jpn. Rev. Clin. Ophthalmol. 66:817-854.
- Simeon, V.: Comparison of biochemical properties of cholinesterases (in Croatian), Arh. Hig. Rada Toksikol. 23 (1972):29-39.
- Simeon, V. 1974a. Effect of acetylcholine on inhibition of cholinesterases by acylating inhibitors. Arh. Hig. Rada Toksikol. 25:51-56.
- Simeon, V. 1974b. Michaelis constants and substrate inhibition constants for the reaction of acetylthiocholine with acetylcholinesterase and cholinesterase. Croat. Chem. Acta 46:137-144.

- Simeon, V., and Reiner, E. 1973. Comparison between inhibition of acetylcholinesterase and cholinesterase by some N-methyl and NN-dimethyl-carbamates. Arch. Hig. Bada Toksikol. 24:199-206.
- Simeon, V., Reiner, E., and Vernon, C. A. 1973. Effect of temperature and pH on carbamylation and phosphorylation of serum cholinesterases: Theoretical interpretation of activation energies in complex reactions. Biochem. J. 130:515-524.
- Sivitskaya, I. I. 1974. Sostojanie organs zrenija u lis rabotajvceih s tetrametiltiuram disulfidom (TMTD). Oftalmol. Zh. 28:286.
- Sivitskaya, I. I., and Alekseev, M. S. 1973. Klinika i sostojaniye funktsij glaza pri vozdejstvii polikloropirena. Vestn. Oftalmol. 3:74-77.
- Skrinjaric-Spoljar, M., Simeon, V., and Reiner, E. 1973. Spontaneous reactivation and aging of dimethyl phosphorylated acetylcholinesterase and cholinesterase. Biochim. Biophys. Acta 315:363-369.
- Stofanec, S., Stengl, B., and Vasilic, S. 1976. Quantitative determination of organophosphorus pesticides by thin-layer densitometry. J. Chromatogr. 124:(1)127-133.
- Stubbs, J. L., and Fales, J. T. 1960. A capillary sampling technique for the determination of cholinesterase activity in red cells and plasma. Am. J. Med. Technol. 26:25-32.
- *Suga, K. 1972. Environmental pollution and eye damage. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol. 66:537-539. From: Health Aspects Pestic. Abstr. Bull. 5(1972) no. 72-2145.
- Suzuki, H., and Ishikawa, S. 1974. Ultrastructure of the ciliary muscle treated by organophosphate pesticide in beagle dogs. Br. J. Ophthalmol. 58:931-940.
- Tabershaw, I. R., and Cooper, W. C. 1966. Sequelae of acute organic phosphate poisoning. J. Occup. Med. 8:5-20.
- Takaku, I. 1972. Studied report of children who have eye diseases due to organophosphate pesticides in Nagasaki Prefecture. Ganka Rinsho Iho (Jpn. Rev. Clin. Ophthalmol. 66:820-823. From: Health Aspects Pestic Abstr. Bull. 6(1973) no. 73-0619.

- Tamura, G., and Mitsu, Y. 1975. Organophosphorus pesticides as a cause of myopia in school children: An epidemiological study. Jpn. J. Ophthalmol. 19:250-253.
- *Tamura, G., and Mitsu, Y. 1975. Relationship between consumption of pesticides and chronological changes in myopia in school children in Takushima Prefecture Hinsho Ganka (Clin. Ophthalmol.) 29:51-55. From: Pestic. Abstr. 8(1975) no. 75-1893.
- **Taneda, M., Oguchi, T. and Ishikawa, S. 1974. EEG study of OP poisoning with special emphasis on REM sleep and narcolepsy. Clin. Neurophysiol. 16:476-482.
- Thompson, W. R. 1947. Using of moving averages and interpolation to estimate median-effective dose Bacteriol. Rev. 11:115-145.
- Tokoro, T. 1973. On the long term observations on the refraction and the intraocular pressure of dogs experimentally and chronically intoxicated by organophosphorus insecticide. Nippon Ganka Gakkai Sesshi (Acta Soc. Ophthalmol. Jpn.) 77:1237-1245. From: Pestic. Abstr. 7(1974) no. 74-0920.
- *Tokoro, T., Suzuki, K., Otsuka, J., and Suzuki, H. 1973. The changes of refraction and ophthalmological pressure of experimental chronically intoxicated beagles by an organophosphorus pesticide. Nippon Ganka Gakkai Sesshi (Acta Soc. Ophthalmol. Jpn.) 77:40. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-2498.
- *Tsuchiya, T. 1973. A report on the state of organophosphorus pesticide application and the cases considered to be intoxication by the pesticide in Miyazaki district. Hinsho Ganka (Clin. Ophthalmol.) 27:1166. From Pestic. Abstr. 7(1974) no. 74-1611.
- Tsukahara, I. 1972. Examination for chronic pesticide poisoning based on examinations of visual acuity, eyegrounds and EPG. Ganka Hinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 66:838-848. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-0824.
- Turina, S., Klossing, L., and Jamnicki, V. 1974. Area determination under chromatographic curves using the Monte Carlo method. Chromatographia 7:(4)303-304.
- Uchida, T. 1974. A case of retinohoroid pathosis due to chronic intoxication by organophosphorus pesticides. Ganka Hinsho Iho (Jpn. Rev. Clin. Ophthalmol.) 68:191-192. From: Pestic. Abstr. 7(1974) no. 74-1106.

- Ueda, K., Nishimura, M., Maebashi, H., Miyasaki, I., Ogita, S., and Sawamura, A. 1973. Experimental chronic intoxication induced by organophosphorus pesticides in beagles. Nippon Nosen Igakkai Shishi (J. Jpn. Assoc. Rural Med.) 21:98-99. From: Environ. Abstr. 6(1973) no. 73-0390.
- Uga, S., Ishikawa, S., and Mukuno, K. 1976. Histopathological study of the canine optic nerve and retina induced by organophosphate pesticides. Nippon Ganka Gakki Shishi (Acta Soc. Ophthalmol. Jpn.) From: author's manuscript.
- Uji, J. 1973. Pollution disasters in Japan. Lakartidningen 69:2789-2796. From: Health Aspects Pestic. Abstr. Bull. 5(1973) no. 73-2512.
- Ueno, M. 1973. Organophosphorus poisoning. Shinkei Kenkyu No Shimpo (Adv. Neurol. Sci.) 16:892-901. From: Health Aspects Pestic. Abstr. Bull. 6(1973) no. 73-2427.
- Upholt, W. M., Quinby, G. E., Batchelor, G. E., and Thompson, J. P. 1956. Visual effects accompanying induced miosis. AMA Arch. Ophthalmol. 56:128-134.
- U.S. Department of Health, Education and Welfare 1969. Report of the Secretary's Commission on Pesticides and Their Relationship to Environmental Health, Parts I and II.
- Vandekar, M., and Svetlicic, B. 1968. Observations on the toxicity of three anticholinesterase insecticides in a village. Scale trial and comparison of methods used for determining cholinesterase activity. Arch. Hig. Bada Toksikol. 17:135-150.
- Voss, G., and Machese, R. 1970. Red cell and plasma cholinesterase activities in microsamples of human and animal blood determined simultaneously by a modified acetylcholine/DTNB procedure. Toxicol. Appl. Pharmacol. 16:764-772.
- Warnick, S. I., and Carter, J. E. 1972. Some Findings in a study of workers occupationally exposed to pesticides. Arch. Environ. Health 25:265-270.
- Weil, S. C. 1952. Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. Biometrics 8:249-263.
- Weintraub, M. I., Ribner, J. A., and Gallagher, B. B. 1971. Transient internal ophthalmoplegia. Arch. Ophthalmol. 86:595-598.

- Wilhelm, K. 1968. Determination of human plasma cholinesterase activity by adapted Ellman's method. Arch. Hig. Rada Toksikol. 19:199-207.
- Wilhelm, K.: Variations in blood cholinesterase activities in nonexposed humans, 18th Int. Congress on Occupational Health, Brighton 1975, Abstr. p. 180.
- Wilhelm, K., Plestina, R.: The influence of previous carbamylation on cholinesterase activity in rats poisoned with some organophosphorus compounds (in Croatian), 1st Yugoslav Congress of Toxicologists, Herceg-Novi 1974, Abstr. no. 83.
- Wilhelm K., Plestina, R., Svetlicic, B.: The toxicity of Etkatin and its action on occupationally exposed workers (in Croatian), Meeting of Chemists of Croatia, Zagreb 1973, Abstr. p. 465.
- Wilhelm, K., and Reiner, E. 1973. Effect of sample storage on human blood cholinesterase activity after inhibition by carbamates. Bull. WHO 48:235-238.
- Wilhelm, K., Vandekar, M., and Reiner, E. 1973. Comparison of methods for measuring cholinesterase inhibition by carbamates. Bull. WHO 48:41-44.
- Wilson, I. V., Hatch, M. A., and Ginsburg, S. 1960. Carbamylation of acetylcholinesterase. J. Biol. Chem. 235:2312-2315.
- Wilson, T. H., and Wiseman, G. 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 123-116-125.
- Witter, R. B. 1963. Measurement of blood cholinesterase. Arch. Environ. Health. 6:537-543.
- WHO Expert Committee on Insecticides 1963. Recommended method for vector control. Tech. Rept. ser. no. 265.
- Yamasaki, H., Shimazaki, K., Kurosawa, K., and Suzuki, A. 1975. Electron microscopic study on the subacute toxicity of organophosphorus pesticides. Nippon Hosen Igakkai Shishi (J. Jpn. Assoc. Rural Med.) 24:1-7. From: Pestic. Abstr. 8(1975) no. 75-1972.

Savva, N. I. 1973. Iamneniia organa zreniia u kolhoznikov;
rabotajuscin s pesticidami. Vestn. Oftalmol. 3:80-83.

Note:

- * Very general abstract - Some results were published in more than one paper; only one paper reviewed;
- ** Article not accessible, even as summary or abstract.

APPENDIX A

INTERNATIONAL MEETING ON CHOLINESTERASES AND CHOLINERGIC RECEPTORS

The Institute for Medical Research and Occupational Health, Zagreb, Yugoslavia, organized an International Meeting on Cholinesterases and Cholinergic Receptors, which was held in Split from the 1st to the 5th of April 1973. The meeting was sponsored by the Yugoslav Academy of Sciences and Arts, and received substantial support from the Scientific Research Council of the Republic of Croatia.

The aim of the meeting was to discuss the molecular properties of cholinesterases and cholinergic receptors. It was felt that an informal exchange of views would be extremely desirable for future developments in that field, as no such meeting was held since the two macromolecules had been purified and isolated. In this aim, the meeting was fully successful and excelled in a stimulating working atmosphere to which everyone contributed. During the meeting it became obvious that great interest exists in models for the binding sites of cholinesterases, and a general discussion was organized on the last day, summarizing old and new concepts on that particular aspect of research.

The participation in the meeting was by invitation through the Organizing Committee which consisted of the following members: W. H. Aldridge (Great Britain), E. DeRobertis (Argentina), Edith Heilbronn (Sweden), G. Kato (Canada), I. A. R. Main (USA), J. Massoulié (France), D. B. Millar (USA), R. D. O'Brien (USA), Nisa Reiner (Yugoslavia) and A. G. Supancic (Yugoslavia). Twenty-nine papers were presented. The total number of participants was 45.

The Proceedings of the meeting are published as No. 1, (XII + 144 pp), Vol 47 of Croatica Chemica Acta and also in the form of a book as an offprint of the same issue of the journal. The editor of the Proceedings was N. Reiner, and the subeditors were M. Skrinjaric-Spoljar and V. Simson. The Proceedings comprise 28 full-length papers and the discussion following each paper. Two participants did not send their manuscripts and one paper was included which was not read. The Proceedings also included a general discussion on models for the ligand binding sites of cholinesterases.

The following are the titles and authors of the papers:

Molecular Forms of Acetylcholinesterase (J. Massoulié, R. Bon, F. Rieger, and M. Vigny), Molecular Structure and Catalytic Activity of Membrane-bound Acetylcholinesterase from Electric Organ Tissue of the Electric Eel (I. Silman and Y. Dudai), Comparative Studies on the Molecular Properties of Purified Acetylcholinesterase from Human Erythrocytes and from the Electric Organ of *Electrophorus Electricus* (U. Bredbeck, P. Ott, and Thérèse Wiedmer), Properties of the Soluble and Membrane-bound Forms of Acetylcholinesterase Present in Pig Brain (D. T. Plummer, C. A. Reavill and C. H. S. McIntosh), Survey of Major Points of Interest about Reactions of Cholinesterases (W. H. Aldridge), Catalysis by Acetylcholinesterase. The Rate-limiting Steps Involved in the Acylation of Acetylcholinesterase by Acetic Acid Esters and Phosphorylating Agents (T. L. Rosenberry), Spectroscopic Studies of Ligand Interactions with Acetylcholinesterase (P. Taylor, J. Iwebuga-Mukasa, H. Berman, and S. Lai), Interaction of Ligand Binding Sites of Acetylcholinesterase (G. Messer and D. S. Sigman), The Reaction Mechanism of Butyrylcholinesterase (K.-B. Augustinsson and H. Erikson), Alkylating Reactions of Horse Serum Cholinesterase (Ljubica Kramarić), Methanesulphonyl Fluoride: A Probe of Substrate Interactions in the Catalytic Site of Acetylcholinesterase (R. M. Krupka), Characterisation of the Active Site of Acetylcholinesterases by Application of Sterically Modified Acetylcholine Homologues (W. H. Hopff, G. Riggio, A. Hofmann and P. G. Wasser), Kinetic Study of the Effect of Substrates on Reversible Inhibition of Cholinesterase and Acetylcholinesterase by two Coumarin Derivatives (Elsa Reiner and Vera Simeon), Non-competitive Inhibition of Flyhead Acetylcholinesterase by Oxime Carbamates, Kinetic Evidence for Non-productive Binding to the Catalytic Site (P. J. Jewess and N. R. McFarlane), The Comparative Biochemistry of Mammalian and Insect Acetylcholinesterase with Reference to the Selective Inhibition by Organophosphates and Carbamates (R. Hallenbrand and R. M. Krupka), Structural Changes in Acetylcholinesterase under the Influence of Some Ligands (M. R. Pavlic), The Use of Affinity Gels for the Study of the Ligand Binding Properties of Mammalian Acetylcholinesterase (G. Hellunger and B. Niklasson), Regulatory Properties of Membrane Bound Acetylcholinesterase from Red Cell Ghosts (H. Kuhn), Inhibition of Acetylcholinesterase by N-Alkylpyridinium and N-Alkylpyridinium-2-aldoxime Salts (L. P. A. de Jong and G. S. Wolring), Effects of Natural Polyamines on Membrane-bound and Solubilised Acetylcholinesterase of Human Red Cells (A. Kossorotow and H. U. Wolf), Current Research on the Nature of Cholinergic Receptors (Edith Heilbronn), Heterogeneities in Acetylcholine Receptor from Torpedo Species (R. D. O'Brien and R. E. Gibson), Structure and Function of the Acetylcholine Receptor (M. E. Eldefrawi and Amira T. Eldefrawi), Actions of Histriocotoxin on Acetylcholine Receptors (G. Rato, M. Glavinović, J. Henry,

K. Krnjevic, E. Pull and B. Tattrie), Purification of Acetylcholine Receptor Enriched Membranes by Use of Affinity Partitioning (S. D. Flanagan, P. Taylor, and S. H. Barondes), Isolation of a Housefly Head Protein Fraction that Exhibits High Affinity Binding of Cholinergic Ligands (P. J. Jewess, H. S. Clarke, and J. P. Donnellan), Regulatory Properties and Cooperativity of Membrane Bound Muscarinic Receptors of Intestinal Smooth Muscle Cells (Bida Fuhnen-Clausen), Cholinergic Muscarinic Receptor: Biochemical and Light Autoradiographic Localization in the Brain (H. I. Yamamura, A.-J. Chang, M. J. Kuhar, and S. H. Snyder), Immunization of Rabbits with Purified Nicotinic Acetylcholine Receptor (Ch. Mattsson and Edith Heilbronn), The Acetylcholine Receptor of the Mammalian Neuromuscular Junction (S. A. Barnard).

APPENDIX B

SUMMARIES OF B.S. AND M.S. THESES

V. Bradamante-Simic: The Effect of Anticholinesterase Pesticides in Occupationally Exposed Workers, M.S. Thesis, University of Zagreb (1975).

The effects of anticholinesterase insecticides were studied in workers employed in the production and formulation of insecticides as well as in workers who apply them in agriculture.

Acetylcholinesterase and cholinesterase activity was followed in workers during the working season and the onset of symptoms and signs of poisoning following exposure to anticholinesterase insecticides were observed.

In addition to these parameters the mode and duration of exposure and the types of insecticides to which workers were exposed in the course of work were determined. As both industrial and agricultural workers were exposed to a combination of insecticides it was not possible to ascertain which insecticide was predominant in producing a biochemical lesion and/or cholinergic symptoms.

The studies in workers formulating the insecticides were carried out from 1971 to 1974, while studies in agricultural workers were done in 1973 and 1974.

The usefulness of protective devices during the production and formulation of insecticides has been confirmed. It was also found in the same workers that a long-term exposure to organophosphorus insecticides does not induce changes in the differential blood picture.

K. Fick: The Persistence of some Organophosphorus Pesticides in Aqueous Media, B.S. Thesis, University of Zagreb (1975).

The aim of this work was to find a suitable procedure for the accumulation of organophosphorus pesticides present as micropollutants in aqueous media. The efficiency of extraction with different solvents and solvent systems was compared with the efficiency of adsorption on the activated carbon microcolumn. The latter procedure was utilized in the studies of the persistence of some organophosphorus pesticides added to river water

samples. Some possibilities for simultaneous determination of dichloroves and trichlorphen by reaction gas chromatographic analysis were investigated.

Gj. Koberšček: Kinetics of Acetylcholinesterase Inhibition by 7-O-Coumarin Derivatives, M.S. Thesis, University of Zagreb (1975).

The kinetics of inhibition of acetylcholinesterase by [0,0,-bis(8-chloroethyl)0-(3-chloro-4-methyl-7-coumarinyl) phosphate] (haloxon) and 3-chloro-7-hydroxy-4-methylcoumarin was studied at 5°C in phosphate buffer pH 7.4. The substrate was acetylthiocholine.

Haloxon reacts with acetylcholinesterase irreversibly (rate of phosphorylation $k = 9.90 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$) the enzyme-haloxon complex is $3.64 \text{ } \mu\text{M}$ (calculated from the rate of phosphorylation) and $3.49 \text{ } \mu\text{M}$ (calculated from the kinetics of competition between haloxon and acetylthiocholine).

3-Chloro-7-hydroxy-4-methyl coumarin inhibits the enzyme reversibly, with dissociation constant $K(I) = 1.1 \text{ } \mu\text{M}$.

The substrate-inhibition constant K_{si} for acetylthiocholine is 3.0 mM (obtained from the activity- pH curve). From the kinetics of reversible inhibition by haloxon and 3-chloro-7-hydroxy-4-methyl coumarin the dissociation constants for the enzyme-substrate reaction are $K(S) = 2.50 \text{ mM}$ and 1.26 mM , respectively. Comparing the K_{si} and $K(S)$ constants it appears that the inhibitors react at the same site of acetylcholinesterase like the substrate, when it inhibits the enzyme. This site is assumed to be an allosteric site of acetylcholinesterase.

B. Krauthacker: Stability of 0,0-Dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (Metrifonate) in Aqueous Solutions and the Mechanism of Inhibition of Cholinesterases in Metrifonate Solutions, M.S. Thesis, University of Zagreb (1974).

The mechanism of inhibition of cholinesterases in solutions of 0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (metrifonate) was studied in 0.1 M phosphate buffer pH 7.4 at 25°C and 37°C. It was shown that the inhibition of cholinesterases is not caused by metrifonate, but by 0,0-dimethyl 2,2-dichlorovinyl phosphate (DDVP) which was formed by nonenzymic rearrangement from metrifonate. This result was obtained from a comparison between the kinetics of enzyme inhibition and the kinetics of the nonenzymic rearrangement from metrifonate which was measured polarographically. The enzyme preparations, used in this work were: acetylcholinesterase (E.C. 3.1.1.7) of rat brain and bovine erythrocytes and cholinesterase (E.C. 3.1.1.8) of human horse and rat serum.

D1. Meyar: Decomposition of O,O-Dimethyl 2,2-dichlorovinyl-phosphate (DDVP) in Rabbit Serum, B.S. Thesis, University of Zagreb (1975).

The rate of DDVP decomposition in rabbit serum was measured in 0.1 M phosphate buffer pH 7.4, at 25°C and 37°C by thin-layer densitometry at four different DDVP concentrations.

The reaction did not follow the Michaelis kinetics at either temperature. At 25°C the rate of decomposition was 0.10 $\mu\text{mol min}^{-1} \text{ ml}^{-1}$ serum (DDVP = 10.0 mM). At 37°C the rate was about five times higher.

The rate of spontaneous decomposition of DDVP was also measured by thin-layer densitometry, and the results agreed with those obtained by other methods.

Only one product containing chlorine was observed either in the enzymic or in spontaneous degradation of DDVP indicating that decomposition consisted in the hydrolysis of the ester bond of the P-O-CH=CHCl moiety.

B. Radic: Determination of Organophosphorus Pesticides in Biological Material by Means of Thin-Layer Chromatography. M.S. Thesis, University of Zagreb (1975).

A method for simultaneous determination of metrifonate and dichlorvos in biological material has been developed.

Due to the complex structures of compounds, not only in the original samples, but also in the extracts, thin-layer chromatography was chosen because it enables separation and quantitative determination of these compounds.

Samples of blood, brain and liver homogenates of experimental animals were used as biological material.

Calibration lines for the quantitative determination by direct measurement on the thin-layer had been made, and the method was applied to animals treated with metrifonate and dichlorvos. Absorption, distribution and persistence of examined compounds were followed after intravenous administration.

It has been established that metrifonate decomposes into dichlorvos, because after intravenous injection of metrifonate it was found that dichlorvos was also present in the blood.

The results for dichlorvos obtained by thin-layer chromatography were compared with those using gas chromatography.

2. Vasilic: Quantitative Determination of Organophosphorus Pesticides by Thin Layer Densitometry, M.S. Thesis, University of Zagreb (1976).

A method for a quantitative thin-layer chromatographic analysis of organophosphorus pesticides is described. The spots visualized with silver nitrate or by enzyme inhibition were quantitatively evaluated by direct densitometric measurements of chromatograms.

The detection with silver nitrate, although neither specially selective nor sensitive, proved to be useful for the determination of dichlorvos and trichlorfon present simultaneously in concentrations of 0.1 - 1 μg and 1 - 4 μg /per 10 μl respectively.

3. Sima: Total Organic Carbon Determination in Water, M.S. Thesis, University of Zagreb (1976).

A method has been developed for the determination of total organic carbon present as a pollutant in water. An apparatus for microanalysis of carbon and hydrogen in organic compounds has been modified to enable a continuous flow of water sample into the combustion tube, the latter being packed with copper oxide and heated to 700°C.

The amount of carbon dioxide obtained by combustion of the polluted water sample in a stream of oxygen has been determined gravimetrically. The water of the sample and the water produced by the pyrolysis of the organic matter was eliminated by condensation in a trap connected to the end of the combustion tube.

The results of carbon determinations (total carbon, organic and inorganic carbon), of the following are discussed: aqueous solutions of pure organic compounds of known composition; organic and inorganic compounds present simultaneously in aqueous solution, and in samples of waste waters and surface waters.

The amount of sample is 1 - 5 ml. The analysis can be completed in roughly one hour. The reproducibility of the results is shown to be ± 2 ppm of carbon. The lower limit of carbon content is not critical, as it can be widened by dilution of the sample.

APPENDIX C

LIST OF PUBLICATIONS SINCE 1972

PAPERS

1. Brevenker, V., Pisk R., Stipčević, M., Stengl, B.: The fate of pesticides in aquatic environment; I. The persistence of some organophosphorus pesticides in river water, *Arch. hig. rada* 26 (1975) 257-266.
2. Plastina, R., Davis, A., Bailey, D. R.: Effect of metrifonate on blood cholinesterases in children during the treatment of schistosomiasis, *Bull. Wild Hlth Org.*, 46 (1972) 747-759.
3. Plastina, R., Svetličić, B.: Toxic effects on two carbamate insecticides in dogs, *Arch. hig. rada*, 24 (1971) 217-225.
4. Plastina, R.: Toxicology of some anticholinesterases used in disinsection (in Croatian), *Pomorska biblioteka*, 26 (1975) 689-695).
5. Reiner, E., Buntić, A., Tadak, M., Simeon, V.: Effect of temperature on the activity of human blood cholinesterases, *Arch. Toxicol.*, 32 (1974) 347-350.
6. Reiner, E., Krauthacker, R., Simeon, V., Škrinjarčić-Spoljar, M.: Mechanism of inhibition *in vitro* of mammalian acetylcholinesterase and cholinesterase in solutions of O,O-dimethyl 2,2,2-trichloro-1-(2-ethoxyethyl) phosphonate (trichlorophen), *Biochem. Pharmacol.*, 24 (1975) 717-722.
7. Reiner, E., Simeon, V.: Kinetic study of the effects of substrates on reversible inhibition of cholinesterase and acetylcholinesterase by two coumarin derivatives, *Croat. Chem. Acta* 47 (1975) 321-331.
8. Simeon, V.: Effect of acetylcholine on inhibition of cholinesterases by acylating inhibitors, *Arch. hig. rada*, 25 (1974) 51-56.

9. Simeon, V.: Michaelis constants and substrate inhibition constants for the reaction of acetylthiocholine with acetylcholinesterase and cholinesterase, *Croat. Chem. Acta*, 46 (1974) 137-144.
10. Simeon, V., Reiner, E.: Comparison between inhibition of acetylcholinesterase and cholinesterase by some N-methyl and NN-dimethyl-carbamates, *Arh. hig. rada*, 24 (1973) 199-206.
11. Simeon, V., Reiner, E., Vernon, C. A.: Effect of temperature and pH on carbamoylation and phosphorylation of serum cholinesterases; Theoretical interpretation of activation energies in complex reactions, *Biochem. J.*, 130 (1972) 515-524.
12. Simeon, V., Skrinjarid-Spoljar, M., Wilhelm, K.: Reactivation of phosphorylated cholinesterases *in vitro* and protecting effects *in vivo* of some pyridinium and quinolinium oximes, *Arh. hig. rada*, 24 (1973) 11-18.
13. Svetlicid, B., Wilhelm, K.: Methods for measuring exposure to anticholinesterase insecticides, *Arh. hig. rada*, 24 (1973) 357-365.
14. Skrinjarid-Spoljar, M., Simeon, V., Reiner, E.: Spontaneous reactivation and aging of dimethylphosphorylated acetylcholinesterase and cholinesterase, *Biochim. Biophys. Acta*, 315 (1973) 363-369.
15. Wilhelm, K., Fleetina, R., Svetlicid, B.: Blood cholinesterase activity in workers exposed to organophosphorus insecticide "Ekatin" (in Croatian), *Arh. hig. rada*, 24 (1973) 107-116.
16. Wilhelm, K., Reiner, E.: Effect of sample storage on human blood cholinesterase activity after inhibition by carbamates, *Bull. Wild Hlth Org.*, 40 (1973) 235-238.
17. Wilhelm, K., Vandekar, M., Reiner, E.: Comparison of methods for measuring cholinesterase inhibition by carbamates, *Bull. Wild Hlth Org.*, 40 (1973) 41-44.
18. Sima, B., Stjepcevic, S., Stefanac, S.: A simple assembly for carbon determination in water, *Croat. Chem. Acta*, 47 (1973) 611-616.

Communications

19. Drevenkar, V., Fink, R., Stipcević, M., Tkalcević, B.: The fate of some organophosphorus pesticides in river water, Scientific Session on Environmental Analysis, Szombathely 1975, (unpublished).
20. Drevenkar, V., Stengl, B., Tkalcević, B., Rešić, J.: Determination of organophosphorus insecticides present as micropollutants in surface waters, Scientific Session on Environmental Analysis, Szombathely 1974, (unpublished).
21. Drevenkar, V., Stengl, B., Stipcević, M.: A method for the determination of organophosphorus pesticides in water, Meeting of Chemists of Croatia, Zagreb 1975, Abstracts, p. 200.
22. Risseljak, B., Wilhelm, K.: Determination of insecticidal carbamates in biological material (in Croatian), 3rd Yugoslav Congress of Pure and Applied Chemistry, Ljubljana 1973., Abstract No. IX-16.
23. Krauthacker, B., Reiner, E., Stipcević, M., Stefanac, S.: Chlorinated hydrocarbons in human blood (in Croatian), 9th Congress of the Yugoslav Physiological Society, Portoroz 1975, Abstract. 125.
24. Poličarić, B., Reiner, E., Becajac, S., Kravavica, S.: Specificity of cholinesterases in *Paramphistomum cervic* (Trematoda) and *Ascaris suum* (Nematoda) (in Croatian), 9th Congress of the Yugoslav Physiological Society, Portoroz 1975, Abstract No. 180.
25. Pleština, R.: Practicability of blood cholinesterase activity measurements for assessment of overexposure to anticholinesterases, 3rd International Congress of Pesticide Chemistry, Helsinki 1974, Abstract Book, No. 133.
26. Pleština, R.: The toxicology of some anticholinesterases used in disinsection (in Croatian), 1st Yugoslav Symposium of Naval Medicine, Trogir 1974, Abstract Book, No. 74.
27. Pleština, R., Fajdetić, T., Matasin, M.: The effect of pH on transport of O,O-dimethyl 2,2,3-trichloro-1-hydroxyethyl phosphonate (metrifonate) through the rat intestinal sac (in Croatian), 9th Congress of the Yugoslav Physiological Society, Portoroz 1975, Abstracts No. 186.
28. Pleština, R., Plukević-Pleština, M., Horvat, S.: Ophthalmological examination of workers exposed to pesticides, 18th International Congress on Occupational Health, Brighton 1975, Abstracts, p. 472.

29. Pleština, R., Rodríguez, F. P., Wilhelm, K.: Influence of inhibition and stimulation of microsomal enzymes on toxic effects of some organophosphorus compounds (in Croatian), 8th Congress of the Yugoslav Physiological Society, Opatija 1973, Abstracts No. 151.
30. Pleština, R., Svetličić, B.: The toxicity of two monomethyl carbamates after different routes of application to dogs (in Croatian), 8th Congress of the Yugoslav Physiological Society, Opatija 1973, Abstracts No. 152.
31. Pleština, R., Wilhelm, K.: The effect of metrifonate (trichlorfon) on rat cholinesterase (in Croatian), 1st Yugoslav Congress of Toxicologists, Herceg-Novi 1974, Abstracts No. 48.
32. Radić, B., Stefanac, S., Wilhelm, K.: Determination of metrifonate and dichlorvos in biological material by thin layer chromatography, Meeting of Chemists of Croatia, Zagreb 1973, Abstracts, p. 261.
33. Reiner, E., Krauthacker, B., Rodríguez, F. P.: Stability of metrifonate and DDVP in aqueous solutions, and inhibition of acetylcholinesterase in metrifonate solution, Meeting of Chemists of Croatia, Zagreb 1973, Abstracts, p. 307.
34. Reiner, E., Krauthacker, B., Simeon, V., Špolja, M.: Mechanism of inhibition of mammalian cholinesterases in trichlorophen solutions, 3rd International Congress of Pesticide Chemistry, Helsinki 1974, Abstract Book, No. 256.
35. Reiner, E., Simeon, V.: Effect of substrates on reversible inhibition of acetylcholinesterase and cholinesterase by two coumarin derivatives, 9th FEBS Meeting, Budapest 1974, Abstracts of Communications, No. 2317.
36. Reiner, E., Simeon, V., Koberšček, B.: Comparison between substrate inhibition of acetylcholinesterase and reversible inhibition by some organophosphorus compounds, 8th FEBS Meeting, Amsterdam 1972, Abstracts of Communications, No. 420.
37. Reiner, E., Škrinjarid-Špoljar, M., Kralić, M.: Cholinesterases in *Metastrongylus apri* (M. apri): Substrate specificity and inhibition by trichlorophen solutions, 10th FEBS Meeting, Paris 1975, Abstracts No. 771.
38. Simeon, V.: Michaelis constants for cholinesterases and the influence of substrate on inhibition by anticholinesterases (in Croatian), 8th Congress of the Yugoslav Physiological Society, Opatija 1973, Abstract No. 94.

39. Simeon, V., Koberhel, D.: Effect of ionic strength and temperature on reversible and progressive inhibition of acetylcholinesterase by some inhibitors, Meeting of chemists of Croatia, Zagreb 1973, Abstracts, p. 305.
40. Simeon, V., Reiner, E.: Effect of temperature on interaction of serum cholinesterases with acylating inhibitors and substrates (in Croatian); 3rd Yugoslav Congress of Pure and Applied Chemistry, Ljubljana 1972, Abstract No. VI-20.
41. Simeon, V., Reiner, E.: Competition of two substrates in their reactions with acetylcholinesterase and cholinesterase, 2. Jahrestagung der Österreichischen biochemischen Gesellschaft, Graz 1975, Programme, p. 3 (unpublished).
42. Siroki, M., Stefanac, S.: Nitrate determination in water by an extraction-visible spectrophotometric method, Scientific Session on Environmental Analysis, Szombathely 1974 (unpublished).
43. Skrinjaric-Spollar, M., Reiner, E.: Spontaneous reactivation and aging of dimethylphosphorylated cholinesterases (in Croatian); 3rd Yugoslav Congress of Pure and Applied Chemistry, Ljubljana 1972, Abstract No. VI-23.
44. Wilhelm, K.: Monitoring of the effects of anticholinesterase pesticides in exposed workers, 3rd International Congress of Pesticides Chemistry, Helsinki 1974, Abstract Book, No. 301.
45. Wilhelm, K.: Variations in blood cholinesterase activities in nonexposed humans, 18th International Congress on Occupational Health, Brighton 1975, Abstracts, p. 160.
46. Wilhelm, K., Plestina, R.: The influence of previous carbamylation on cholinesterase activity in rats poisoned with some organophosphorus compounds (in Croatian), 1st Yugoslav Congress of Toxicologists, Herceg-Novi 1974, Abstract No. 83.
47. Wilhelm, K., Plestina, R., Svulicic, B.: The toxicity of Eshatin and its action on occupationally exposed workers (in Croatian), Meeting of Chemists of Croatia, Zagreb 1973, Abstracts, p. 465.

Books

48. Aldridge, W. N., Reiner, E.: "Enzyme inhibitors as substrates. Interaction of esterases with esters of organophosphorus and carbamic acids", North Holland Publ. Co., Amsterdam (1972) XVI + 320 pp.; First reprint, 1975.

49. Reiner, E. (Editor): "Cholinesterases and cholinergic receptors", Proceedings of the International Meeting, Split 1975, Croatia Chemical Acta, Zagreb (1975), XII + 144 pp.

Reviews

50. Krauthacker, B.: Methods for determination of 0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (DDTHF) (in Croatian); Arh. hig. rada; 26 (1975) 157-169.
51. Simson, V.: Comparison of biochemical properties of cholinesterases (in Croatian); Arh. hig. rada; 23 (1972) 29-39.
52. Wilhelm, K.: Toxicology and prevention of poisoning with some pesticides used for protection of stored food stuff (in Croatian); Bilina sastita; 19 (1978) 87-90 (part I) and 126-129 (part II).

Theses

53. Bradamante-Simić, V.: The effect of anticholinesterase pesticides in occupationally exposed workers (in Croatian); M. Sc. Thesis, University of Zagreb, 1975.
54. Fink, K.: The persistence of some organophosphorus pesticides in aqueous media (in Croatian); B. Sc. Thesis, University of Zagreb, 1975.
55. Kobrshel, D.: Kinetics of acetylcholinesterase inhibition by two coumarin derivatives (in Croatian); M. Sc. Thesis, University of Zagreb, 1975.
56. Krauthacker, B.: Stability of 0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (metrifonate) in aqueous solutions and the mechanism of inhibition of cholinesterases in metrifonate solutions (in Croatian); M. Sc. Thesis, University of Zagreb, 1974.
57. Mrvos, D.: Decomposition of 0,0-dimethyl 2,2-dichloro-vinylphosphate (DDVP) in rabbit serum (in Croatian); B. Sc. Thesis, University of Zagreb, 1975.
58. Radić, B.: Determination of organophosphorus pesticides in biological material by means of thin layer chromatography (in Croatian); M. Sc. Thesis, University of Zagreb, 1975.

59. Vasilčić, S.: Quantitative determination of organophosphorus pesticides by thin layer densitometry (in Croatian), B. Sc. Thesis, University of Zagreb, 1976.
60. Sima, S.: Total organic carbon determination in water (in Croatian), B. Sc. Thesis, University of Zagreb, 1976.